EPIDEMIOLOGICAL ANALYSIS OF FLAVIVIRUS INFECTIONS IN TAMIL NADU WITH SPECIFIC REFERENCE TO JAPANESE ENCEPHALITIS, DENGUE AND WEST NILE VIRUSES. DEVELOPMENT OF A NOVEL DETECTION SYSTEM.

A thesis submitted to

The Tamil Nadu Dr.M.G.R. Medical University

for the award of the degree of

DOCTOR OF PHILOSOPHY

By

V. SENTHILKUMAR, M.Sc., M.Phil.,

DEPARTMENT OF VIROLOGY

KING INSTITUTE OF PREVENTIVE MEDICINE & RESEARCH

GUINDY, CHENNAI - 600 032.

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DECLARATION

I hereby declare that the thesis entitled **"EPIDEMIOLOGICAL ANALYSIS OF FLAVIVIRUS INFECTIONS IN TAMIL NADU WITH SPECIFIC REFERENCE TO JAPANESE ENCEPHALITIS, DENGUE AND WEST NILE VIRUSES. DEVELOPMENT OF A NOVEL DETECTION SYSTEM"** submitted to the Tamil Nadu Dr. M.G.R. Medical University for the award of the degree of Doctor of Philosophy is the original and independent work carried out by me in the Department of Virology, King Institute of Preventive Medicine & Research, Guindy, Chennai-32.

Date: 30.09.15

Place: Chennai.32.

V. SENTHILKUMAR

CERTIFICATE

This is to certify that the thesis entitled **"EPIDEMIOLOGICAL ANALYSIS OF FLAVIVIRUS INFECTIONS IN TAMIL NADU WITH SPECIFIC REFERENCE TO JAPANESE ENCEPHALITIS, DENGUE AND WEST NILE VIRUSES. DEVELOPMENT OF A NOVEL DETECTION SYSTEM"** is based on the results of the work carried out by **Mr.V.Senthilkumar** for Ph.D degree under my supervision and guidance. This work has not been submitted to any degree or diploma of any other university.

Dr.P.Gunasekaran, M.D., M.B.A.,
Director,
King Institute Of Preventive Medicine and Research,
Guindy, Chennai - 600 032.

Date: 30.9.2015

Place: Chennai

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V. SENTHIL KUMAR

CONTENTS

ABBREVIATIONS

µl	-	Microlitre
µg	-	Microgram
AES	-	Acute Encephalitis Syndrome
Ab	-	Antibody
Ag	-	Antigen
ACD	-	Acid citrate dextrose
BABS	-	Bovine albumin borate saline
BBB	-	Blood brain barrier
BSA	-	Bovine serum albumin
BP	-	Base pair
CDC	-	Centre for Disease Control
CFT	-	Complement fixation test
CNS	-	Central nervous system
CPE	-	Cytopathic effect
DEET	-	N,N-diethyl-3-meta-toluamide
DENV-1	-	Dengue virus 1
DENV-2	-	Dengue virus 2
DENV-3	-	Dengue virus 3
DENV-4	-	Dengue virus 4
DF	-	Dengue fever
DHF	-	Dengue hemorrhagic fever

DSS	-	Dengue shock syndrome
ECL	-	Electrochemiluminescence
E. protein	-	Envelope protein
EDTA	-	Ethylene Diamine Tetra Acetic acid
ELISA	-	Enzyme-linked Immunosorbent Assay
ER	-	Endoplasmic reticulum
FCS	-	Fetal calf serum
FITC	-	Fluorescein Isothiocyanate
HA	-	Haemagglutination
HAI	-	Haemagglutination Inhibition
HAU	-	Haemaggluting Units
HRP	-	Horse radish peroxidase
HSV	-	Herpes Simplex virus
IFA	-	Immunofluorescence assay
IgG	-	Immunoglobulin G
IgM	-	Immunoglobulin M
IL - 1	-	Interleukin - 1
IU	-	International Unit
JEV	-	Japanese encephalitis virus
KDa	-	Kilo daltons
Mab	-	Monoclonal antibody
MEGA	-	Molecular Evolutionary Genetics Analysis
M.protein	-	Membrane protein

MEM	-	Minimum essential medium
MIA	-	Micro sphere Immuno assay
Ml	-	Milli litre
NAAT	-	Nucleic Acid Amplification tests
NASBA	-	Nucleic acid sequence based amplification method
NCR	-	Non coding regions
NIMHANS	-	National Institute of Mental Health and Neuro Science
NIV	-	National Institute of Virology
nm	-	Nanometer
NS	-	Non structural protein
nt	-	Nucleotide
OD	-	Optical Density
PBS	-	Phosphate buffered saline
PBST	-	Phosphate buffered saline – Tween – 20
PCR	-	Polymerase Chain Reaction
PRNT	-	Plaque Reduction Neutralization test
RBC	-	Red blood cells
RNA	-	Ribonucleic acid
RPM	-	Revolution Per Minute
RT-PCR	-	Reverse transcription - polymerase chain reaction
SLE	-	St. Louis encephalitis virus
SD	-	Standard Deviation
TBE	-	Tris Borate EDTA

TMB	-	Tetra methyl benzidene
TPVG	-	Trypsin Phosphate Versine Glucose
TNF-α	-	Tumor necrosis factor
VAD	-	Virus Adjusting Diluent
WHO	-	World Health Organization
WNF	-	West Nile fever
WNND	-	West Nile Neuroinvasive disease
WNV	-	West Nile Virus
YF	-	Yellow fever
UIP	-	Universal Immunization Programme

Dedicated to

my beloved

Parents,

Research Guide

and Friends.....

INTRODUCTION

1.1 FLAVIVIRUS

The genus Flavivirus of the family *Flaviviridae* comprises over 70 viruses, many of which, such as the Dengue (DEN) virus, Japanese Encephalitis (JE) virus, West Nile virus (WNV), St. Louis Encephalitis (SLE) virus, and Yellow fever (YF) virus are important human pathogens[1,2]. Flavivirus can be transmitted to humans via either a mosquito or tick vector. The flavivirus genera are of importance to the medical community because they have been found to be the causative agent of many endemic and epidemic illnesses across the world. Japanese Encephalitis virus (JEV) is the most important cause of viral encephalitis in Asia based on its frequency and severity. With the near eradication of poliomyelitis, JEV is now the leading cause of childhood viral neurological infection and disability in Asia[3].

Dengue and its severe and sometimes fatal forms, Dengue hemorrhagic fever and Dengue shock syndrome, alone affect nearly 80 million people a year[4]. As demonstrated in recent out breaks of meningitis by West Nile (WN) virus in Algeria and Romania, viruses of this group sometimes cause serious public health concern in unexpected locations[5].

The first flavivirus discovered to infect human was the Yellow Fever virus, which led to the descriptive naming of this entire family of these viruses[6,7]. The family was subsequently named “means “yellow” in Latin after the associated jaundice that occurs during infection [8]. Since their discovery, the flavivirus persist as causative agents for a wide range of infectious diseases worldwide. However, the most common causes of disease are associated with Japanese Encephalitis group viruses within the flavivirus genus[9,10,11].

There are over 70 various flavivirus species, but not all of them are known to cause human disease. Many time, an individual may be infected with one of these flavivirus species and not even be aware, as an asymptomatic infections are common in regard to flavivirus infections. Severe infection of certain flaviviruses can lead to serious complications, such as inflammation of the brain, hemorrhage and death.

Most of the flavivirus species cause acute encephalitis syndrome in human. The flaviviruses include Japanese Encephalitis virus (JEV) Dengue virus (DENV) and West Nile Virus (WNV) which are the main cause of encephalitis in human[12].

The epidemiology of flavivirus encephalitis is governed by a complex interplay of climatic, entomologic, human behavioural, viral, host factors that are not completely understood and the virus is transmitted naturally among birds in enzootic cycles by bird biting mosquitoes especially the *Culex* genus. Humans become infected inadvertently when they encroach on this cycle, but they are considered "dead-end" hosts because normally they do not have sufficiently high or prolonged viraemia to transmit the virus further. In Asia, pigs as well as birds are important natural hosts for Japanese Encephalitis virus, since these animals are often kept close to human dwellings they serve as amplifying or bridging hosts that transmit the virus to humans [13].

Japanese Encephalitis mostly occurs in areas of South Asia, South East Asia, and the Pacific with transmission of the disease likely to increase in Bangladesh, Cambodia, Indonesia, Laos, Myanmar, North Korea, and Pakistan[5,14,15]. The burden of DF and DHF disease is not very well documented, however in 1998 alone, more than 1.2 million cases were reported to the World Health Organization, with South East Asia, the Western Pacific and more recently the Americas being the most affected regions[16]. West Nile virus is of public health importance and has a wide geographical range that includes portions of New York, Romania Russia Israel[17].

The first outbreak of DHF was recorded in 1963 in Kolkata,[18] since Dengue spreads to all parts of India[19]. The first outbreak of JE occurred in Pondicherry and Vellore (Tamil Nadu)[20] in South India in 1955 and later spread all over the country, including Haryana state in North India[21]. The West Nile virus was first characterized in several outbreaks in the Mediterranean basin in the early 1950s and 1960s[22].

National Status of AES in India

The incidence of acute encephalitis syndrome (AES) has been reported worldwide. Viruses are the most common causative agents of AES, though bacteria, fungi, parasites, and toxins have also been implicated in its etiology. The incidence of AES varies from 0.9 per 100,000 adults in Nigeria, to 185 per 100,000 adults for a rural population in Nepal during an outbreak of Japanese Encephalitis (JE)[23].

In India, it has been estimated that a population of 375 million people residing in 171 endemic districts of 17 states are at a risk of acquiring AES[24]. Approximately 70% of the disease burden is from the Northern State of Uttar Pradesh (UP), which has become an epicentre for this killer disease. In the year 2012 alone, 3,494 patients suffering from AES were admitted to different government hospitals of Gorakhpur and Basti divisions, 588 of who died[25].

Japanese Encephalitis virus (JEV) has been the major and consistent causative agent of AES in UP, annually accounting for approximately 10–15 yrs of the patients[26]. The growth of vector mosquito population is favoured by the accumulation of water and extensive rice cultivation in the Terai region of Eastern UP and other adjoining regions that run parallel to the lower ranges of the Himalayas. Besides JEV, other viruses that have contributed to the high incidence of AES in India include the Dengue virus (DV), Enterovirus, Herpes simplex virus (HSV), Measles virus and Chandipura virus[27] however, the aetiology of AES remains unknown in 68–75% of the patients. An accurate identification of the organism causing AES is essential for surveillance and patient management because some of these infections are preventable or treatable.

AES outbreaks often have a high mortality and hence are a major public health concern in India. Since the first major reported outbreak of AES from Eastern India (Bankura, West Bengal) in 1973[28,29] parts of the country have been devastated by numerous outbreaks with striking regularity. The surveillance for sporadic cases of AES has been limited. Subsequent to early studies from Lucknow (1957–58)[30] and Vellore (1960–61)[31], the Indian Council of Medical Research initiated JEV surveillance in many parts of the country, focusing on mosquito-borne viruses. In

these studies, investigators conducted serological tests and isolated viruses, collecting zoonotic and entomological evidence with an eye towards finding JEV as the aetiological agent. Surveillance studies conducted in the same regions that had experienced prior AES outbreaks reported about one-quarter to one-half of all cases to be seropositive for IgM antibodies against JEV[32-34].

As a result, most outbreaks are presumptively attributed to JEV, before any investigations are initiated. In recent years, investigations into large outbreaks of AES have been negative for JEV (or a flavivirus). Instead outbreaks were found to be due to Rhabdovirus (Chandipura virus)[35] or water-borne enteroviruses. These outbreaks have also occurred in hot and humid seasons, have predominantly affected children, and have had a high case-fatality. Surveillance studies conducted in inter-epidemic period have also found other aetiologies. It needs to be emphasized that in the absence of a definite viral diagnosis, other predictors of aetiology such as clinical features, seasonality and prognosis may not be able to distinguish between aetiologies. While viral diagnosis is tedious, expensive and may not be possible for individual patients, it must be done periodically at population levels to record epidemiological shifts.

Several factors might account for enteroviruses replacing JEV as the major cause of AES. First, JE vaccination campaigns, launched in endemic districts, may have brought about this shift. According to a recent systematic review of AES surveillance studies globally, 2 JE vaccination programmes in developing countries reduce the incidence of JE and bridge the gap between the incidence of AES in developed and developing countries. This observation is supported by epidemiological data which show that the introduction of JE vaccination in endemic regions reduced the overall incidence of AES[36].

Second, it is likely that once the incidence of JE falls either due to vaccination or due to periodic fluctuations in the circulation of JEV or its vector, AES caused by other neuropathogenic aetiological agents are ‘unmasked’, although at a much lower incidence. Advances in molecular diagnostics, viral culture and isolation, as well as use of an extended panel of tests for potential aetiological agents

could be other factors leading to increased frequency of identification of alternative aetiologies.

The emergence of non-JEV aetiologies in outbreaks and surveillance studies directly impacts preventive measures for AES. While vector control programmes and JEV vaccination remain important strategies, the presence of other agents calls for designing and implementing novel preventive strategies that would focus on containment of water-borne enteroviruses and vectors for Chandipura virus. This will need a multisector approach involving health, water resources, sanitation and rural development departments. Recently the thought process on such an approach has been initiated[37].

In addition, need to move from JE surveillance to surveillance for the entire spectrum of AES, so that evidence based public health actions can be planned and carried out. While this review is based on a thorough search of the literature, it has certain limitations. Publication bias is a major limitation because studies with negative or uncertain aetiological outcome might not have been published in biomedical journals. Such technical reports and unpublished documents from national and regional disease control organizations often do not find their way to scientific journals.

Second, earlier researchers seldom used a battery of tests that would include all possible viruses causing AES. Not only did the studies lack consistency, they also differed from one another in respect to the viral diagnostic methods employed, and the range of aetiologies for which diagnostic tests were included. For example, researchers investigating outbreaks of AES were more likely to look for JEV if this virus was also reported from the same region in the past. Third, big outbreaks are more likely to be investigated and reported, and surveillance studies are more likely to be conducted, because they are more likely to impact public health. Lastly, in the recent past India has seen epidemics of Chikungunya and Dengue, which mostly present as fever-arthralgia and fever-rash, respectively.

AIM AND OBJECTIVE

AIM

1. Seroprevalence of antibodies to flaviviruses in the population of Tamil Nadu.
2. Screening of AES patients for flavivirus etiology by RT-PCR.
3. Genetic characterization of the positive samples.
4. Sequencing and Phylogenetic analysis of circulating strains.
5. Development and Standardization of pan-flavivirus detection system (ELISA) using peptide antigen to enable early and economical detection of flavivirus infection.

OBJECTIVE

Dengue, Japanese Encephalitis and West Nile encephalitis, are the common viral diseases associated with high morbidity and mortality. There is not much data available on the seroprevalence of the flavivirus antibodies in the population of Tamil Nadu. A seroprevalence study will give us a clear picture about the exposure of the population to flavivirus.

The initial symptoms of most of the viral infections are similar to each other as well as to some other viral diseases. Making clinical diagnosis, therefore, becomes a challenging task for the clinician. Several studies have been reported on using detection of serum antibodies against flavivirus and the use of specific primers for the diagnosis of flaviviral disease. The use of RT-PCR and ELISA will help to detect the early infection and also if the sample is collected after a week time, thus giving us an accurate picture about the prevalence of flaviviruses.

Genetic analysis of the flavivirus will help us to understand the etiology of the circulating strains. The phylogenetic analysis will reveal the circulating genotypes of the flavivirus[11]. Given that virus genetic diversity may influence

disease severity, the phylogenetic analysis will help in assessing the severity of the disease that the strain will cause and will help in taking precautionary measures.

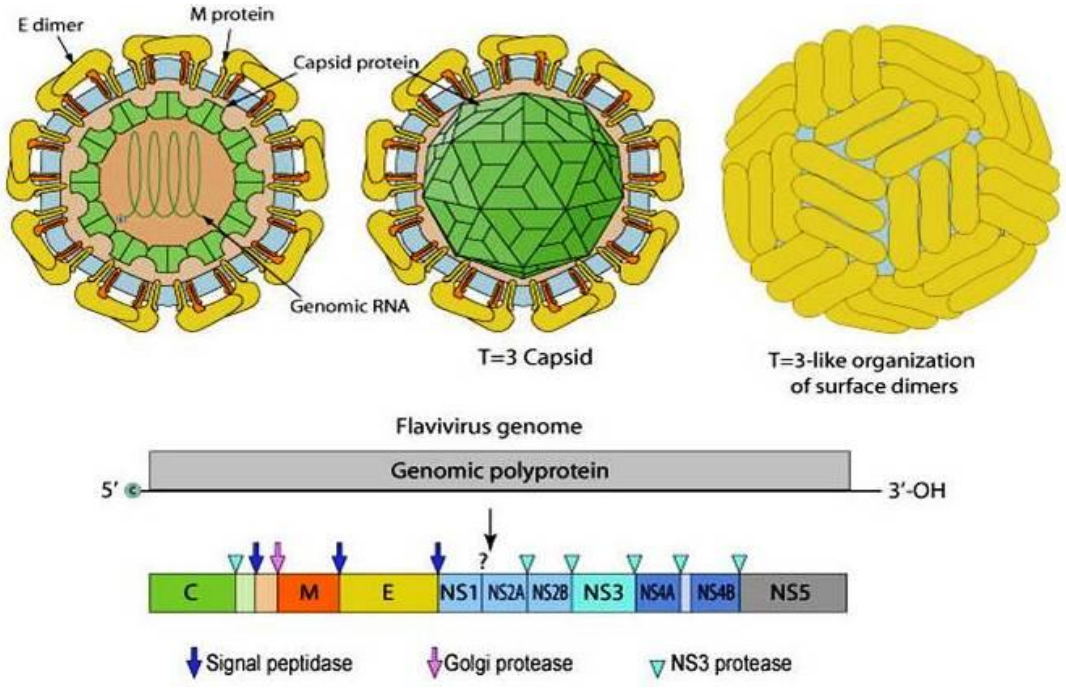
Several diagnostic systems are available for the detection of circulating antibodies in patients serum. However, no gold standard is available for immunological diagnosis of flaviviruses. A field based simple method to use pan-flavivirus detection (ELISA) system may be of great help specifically for low endemic areas to differentiate flavivirus infections from other viral infections[26].

REVIEW OF LITERATURE

3.1 FLAVIVIRUS

Virus is an intra cellular parasite and classified as animal virus and plant virus. It is replicated by lytic cycle and lysogenic cycle in host cell. The AES case mainly denotes either by infected viruses JE, Dengue, West Nile. All these 3 viruses come under the flavivirus. The flavivirus genus comes from the *flaviviridea* family of viruses, which are classified as arthropod-borne viruses. Flavivirus species can be transmitted to humans via either a mosquito or tick vector. They are (+)-sense, single stranded RNA icosahedral viruses that are surrounded by an envelope (Figure 3.1). All flavivirus are similar in size, ranging from about 40-65 nm. Flaviviruses also share a common genome size of about 9500-12500 nucleotides[38,39]. The flavivirus genera are of importance to the medical community because they have been found to be the causative agent of many endemic and epidemic illnesses across the world.

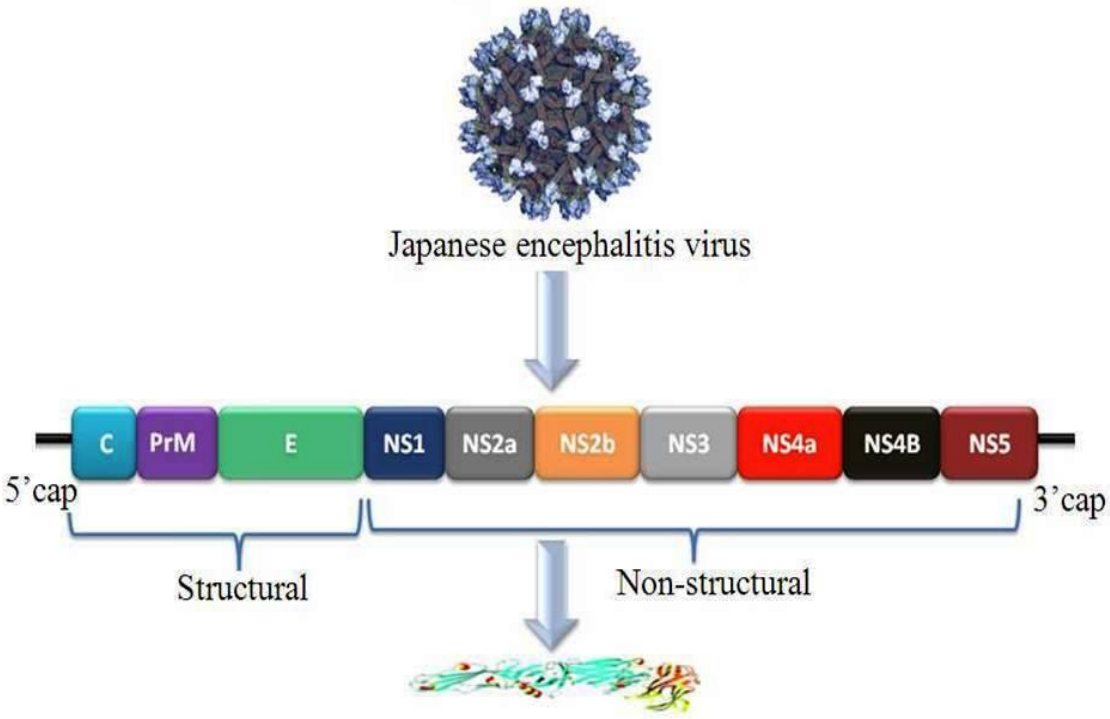
Figure 3.1



3.1.1 Structure of Japanese Encephalitis virus

The causative agent Japanese Encephalitis virus is an enveloped virus of the genus flavivirus. The positive sense single stranded RNA genome is packaged in the capsid which is formed by the capsid protein. The outer envelope is formed by envelope (E) protein and is the protective antigen. It aids in entry of the virus to the inside of the cell. The genome also encodes several nonstructural proteins also (NS1, NS2a, NS2b, NS3, N4a, NS4b, NS5) (Figure 3.2). NS1 is produced as secretary form also. NS3 is putative helices, and NS5 is the viral polymerase. It has been noted that the Japanese Encephalitis virus (JEV) infects the lumen of the endoplasmic reticulum (ER) and rapidly accumulates substantial amounts of viral proteins for the JEV[40,41].

Figure 3.2

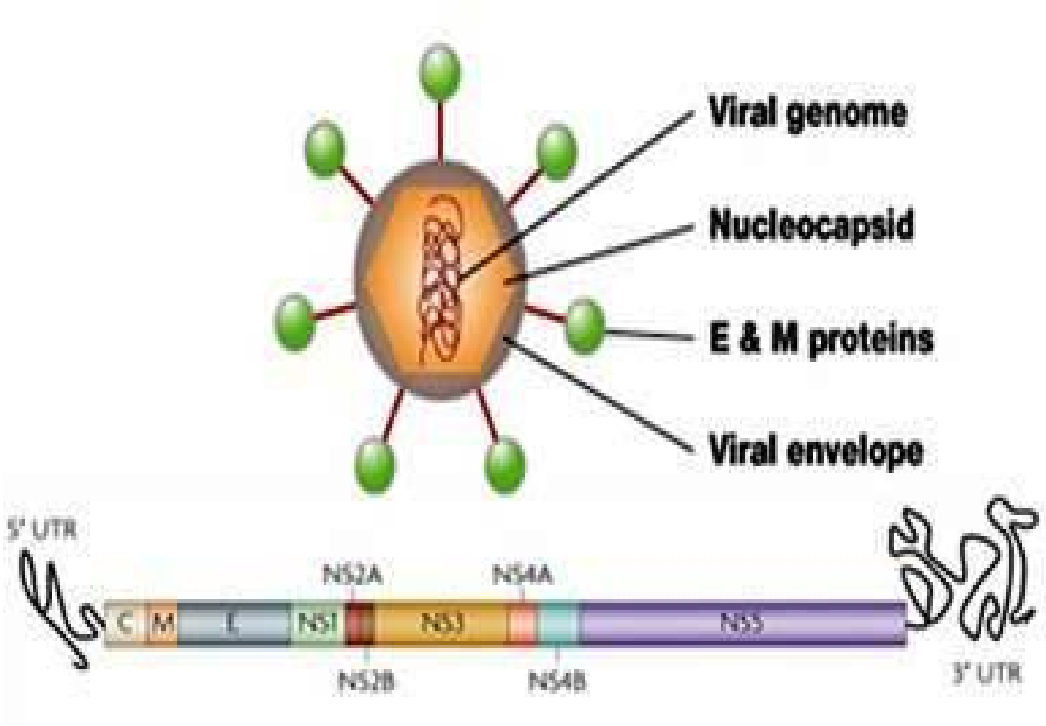


3.1.2 Structure of Dengue virus

Genome of Dengue virus is enveloped, spherical, about 40-50 nm in diameter contains about 11,000 nucleotide bases, which code for the three different types of protein molecules (C, prM and E) that form the virus particle and seven

other types of protein molecules (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) that are only found in infected host cells and are required for replication of the virus (Figure 3.3). It is a Monopartite, linear, ssRNA (+) genome of about 10-12 kb and spherical nucleocapsid lipid bilayer envelope**.** Replication occurs in cytoplasm**.** Have four serotypes (DEN-1, 2, 3, 4)**.** All four serotypes can cause severe and fatal disease. There is genetic variation within each of the four serotypes[42].

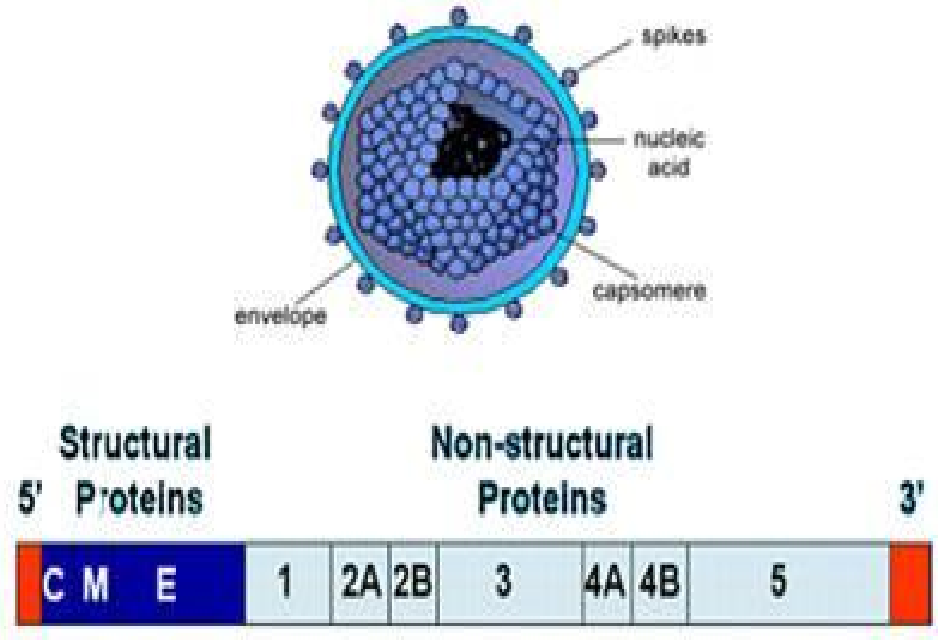
Figure 3.3



3.1.3 Structure of West Nile Virus

West Nile virus (WNV) is a mosquito-borne zoonotic arbovirus belonging to the genus flavivirus in the family *flaviviridae*. Image reconstructions and cryoelectron microscopy reveal a 45–50 nm virions covered with a relatively smooth protein surface. This structure is similar to the Dengue virus. The genetic material of WNV is a positive-sense, single strand of RNA, which is between 11,000 and 12,000 nucleotides long; these genes encode seven nonstructural proteins and three structural proteins (Figure 3.4). The RNA strand is held within a nucleocapsid formed from 12-kDa protein blocks, the capsid is contained within a host-derived membrane altered by two viral glycoproteins[43].

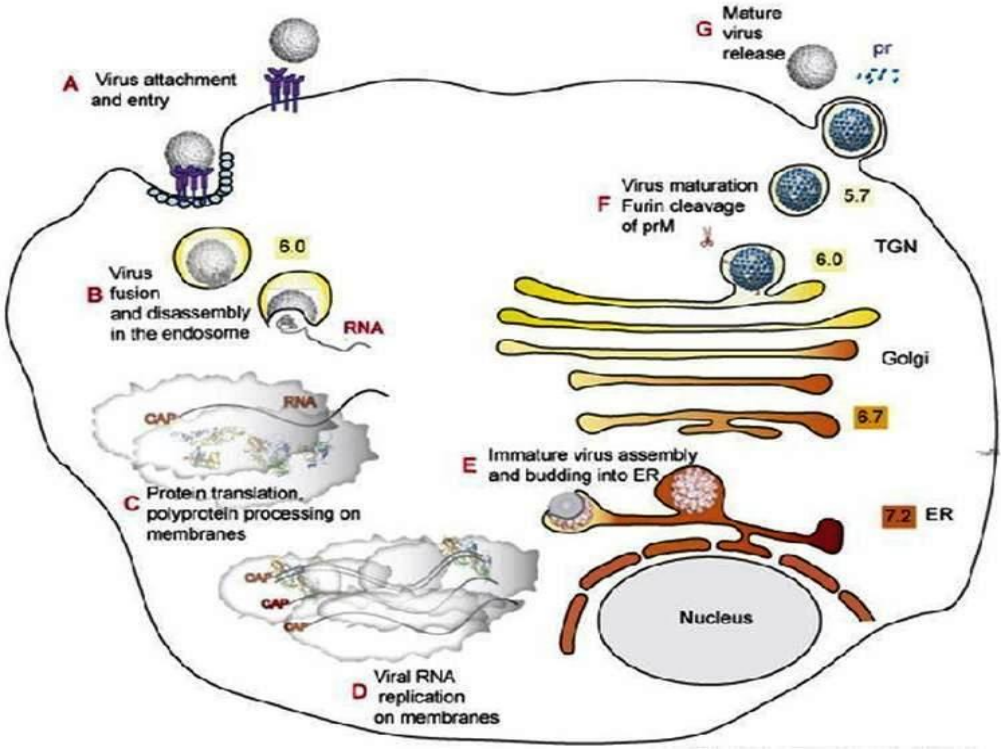
Figure 3.4



3.2 LIFE CYCLE OF FLAVIVIRUS

Viruses belonging to the family *flaviviridae* are considered class 4 viruses within the Baltimore classification scheme. Viruses in this class are positive sense, single stranded RNA viruses which replicate their genome through a partially double-stranded intermediate form. The following sections address the life-cycle of viridae specifically, but they still fall close to the generic category of class 4 viruses (Figure 3.5). The structural organization of flaviviruses and their structural proteins has provided insight into the molecular transitions that occur during the viral life cycle and their stages are virus attachment, entry, and uncoating these section overviews the proteins important to a flavivirus tissue tropism, the method used for entering the cell and the strategies used for signaling viral uncoating. Expression and Biosysnthesis, the various genes of the generic flavivirus genome and the timing related to each gene[44].

Figure 3.5



3.3 Methods of Transmission

There are four categories of ecological and genomic division for flavivirus. Two of these groups use the mosquito as their transmission vector. As stated in the vaccine section, almost half of all flaviviruses use the mosquito vector for transmission. Another group is founded by the flaviviruses using ticks as their vectors, and a final group represents the flaviviruses without a (known) vector. Viruses use only invertebrates as their transmission vectors and how flaviviruses infect primarily vertebrates. Flaviviruses are grouped separate from other *flaviviridae* because of this peculiarity that is, the ability to infect vertebrates and invertebrates as well as their strange methods of transmission[45].

3.3.1 Transmission of Japanese Encephalitis Virus (JEV)

The vertical transmission of JEV refers to transmission of the virus to the next generation of mosquitoes. Vertical transmission probably occurs at oviposition rather than transovarially which might account for the persistence of virus in nature[46]. Vertical transmission of JEV has been reported in 3 strains of *C. tritaeniorrhynchus, C. Pipiens, Aedes albopictus, A. togoi, C. annulus, C. quinquefasciatus and Armigeres subalbatus* mosquitoes.

A high prevalence of JEV antibodies has been documented in pigs, horses and birds and to a lesser extent in cattle, sheep, dogs and monkeys. Pigs and ardecid birds are the most important hosts for maintenance, amplification and spread of JEV. Pigs are the main component in the transmission cycle with respect to human infection whereas herons, egrets and other ardeid birds are important maintenance hosts. JEV infected animals and mosquitoes generally remain asymptomatic, although fatal encephalitis occurs in horses and fetal wastage occurs in swines[47]. These effects on domestic animals have lead to the development of animal vaccines. The domestic animals can get infected but show no evidence of viremia. Rodents appear to be unimportant hosts[48]. Amphibians, reptiles and bats can become infected experimentally and the virus can persist. Pigs are the most important reservoir of JEV because of the following reasons:

(1) High incidence of natural swine infection.

(2) High frequency of viremia in pigs after infected mosquito bite.

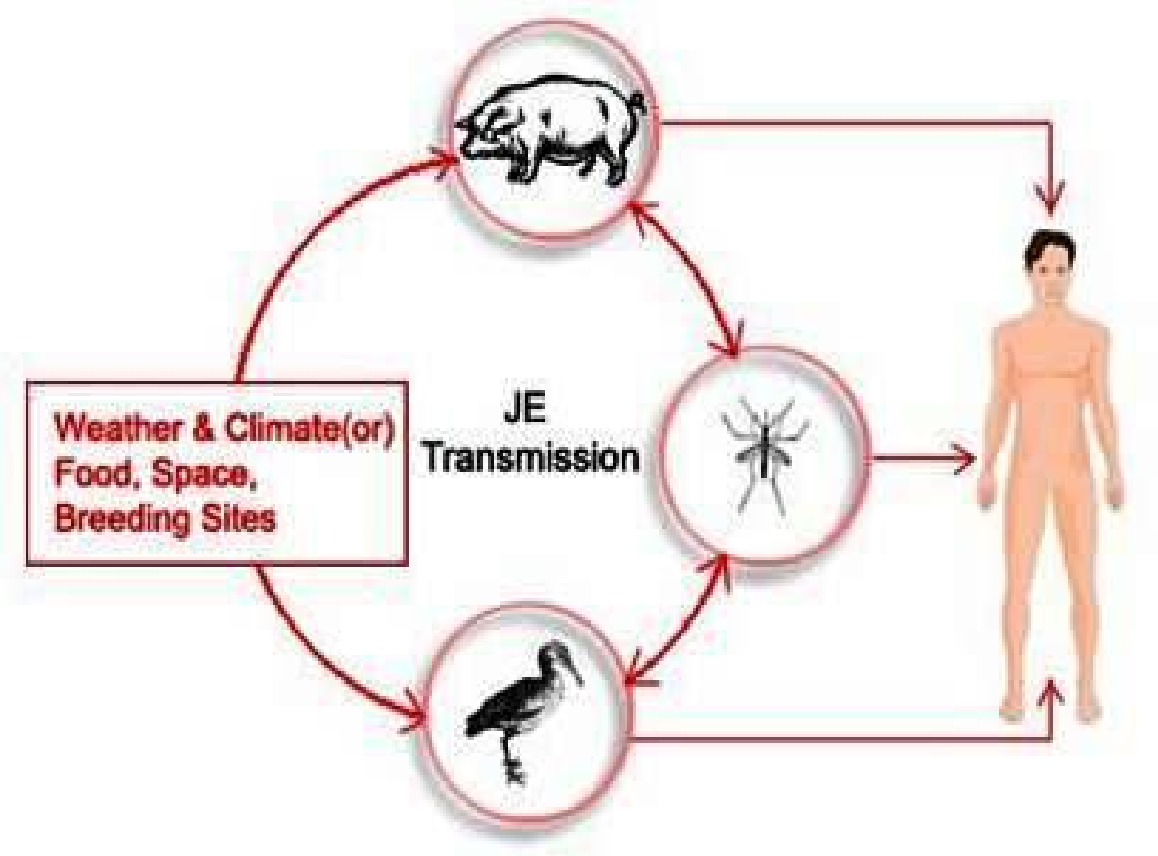
(3) Viremia lasting in high titre for 2–4 days which is adequate to infect *C. tritaeniorrhynchus.*

(4) Transmission of JEV from pig to pig by laboratory reared *C. tritaeniorrhynchus*

(5) Large number of *C. tritaeniorrhynchus* mosquitoes found biting pigs in nature.

(6) Presence of large number of susceptible pigs is replenished each year due to commercial slaughtering of the animals at 10–16 months of age[49].

Figure 3.6: Transmission of JEV



3.3.2 Transmission of Dengue Virus

Susceptible human become infected after being bitten by an infected female *Aedes aegypti* mosquito. Viremia in humans beings toward the end of 4-6 days incubation period and persists until fever abates, which is typically 3-7 days. An uninfected *Ades* mosquito may acquire the virus after feeding during this viremic period. The mosquito has an incubation period 8-12 days before it is capable of transmitting the virus to susceptible individuals. Once infected, mosquito carry the virus for their life span and remain infective for humans[50,51].

The part of the transmission cycle that takes place within the human body.

1. The virus is inoculated into humans with the mosquito saliva.

2. The virus localizes and replicates in various target organs, for example, local lymph nodes and the liver.

3. The virus is then released from these tissues and spreads through the blood to infect white blood cells and other lymphatic tissues.

4. The virus is then released from these tissues and circulates in the blood.

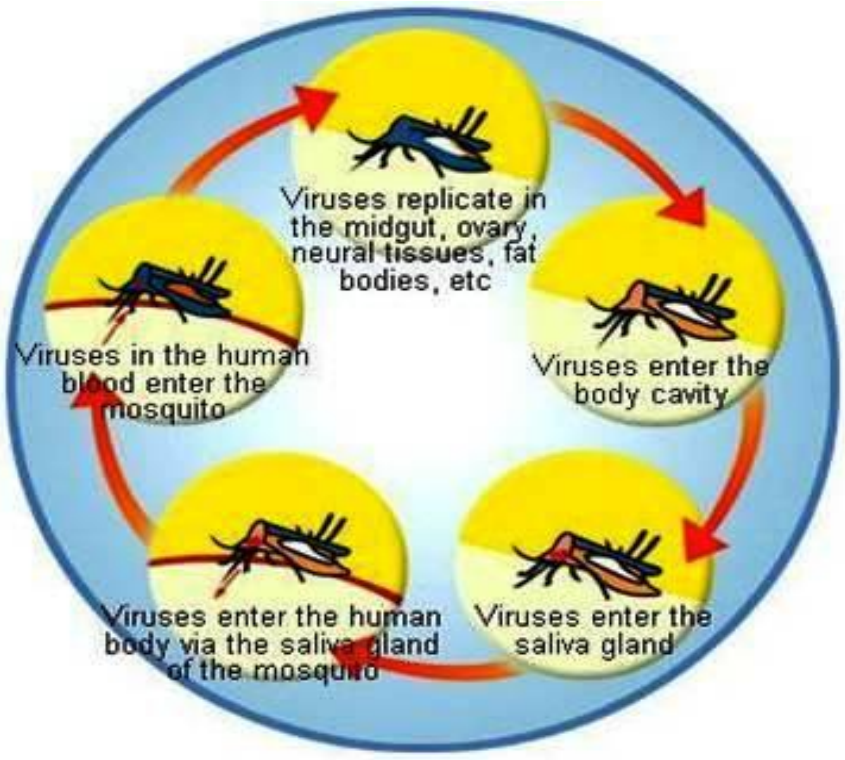
The part of the transmission cycle that takes place within the mosquito.

1. The mosquito ingests blood containing the virus.

2. The virus replicates in the mosquito midget, the ovaries, nerve tissue and fat body. It then escapes into the body cavity, and later infects the salivary glands.

3. The virus replicates in the salivary glands and when the mosquito bites another human, the cycle continues.

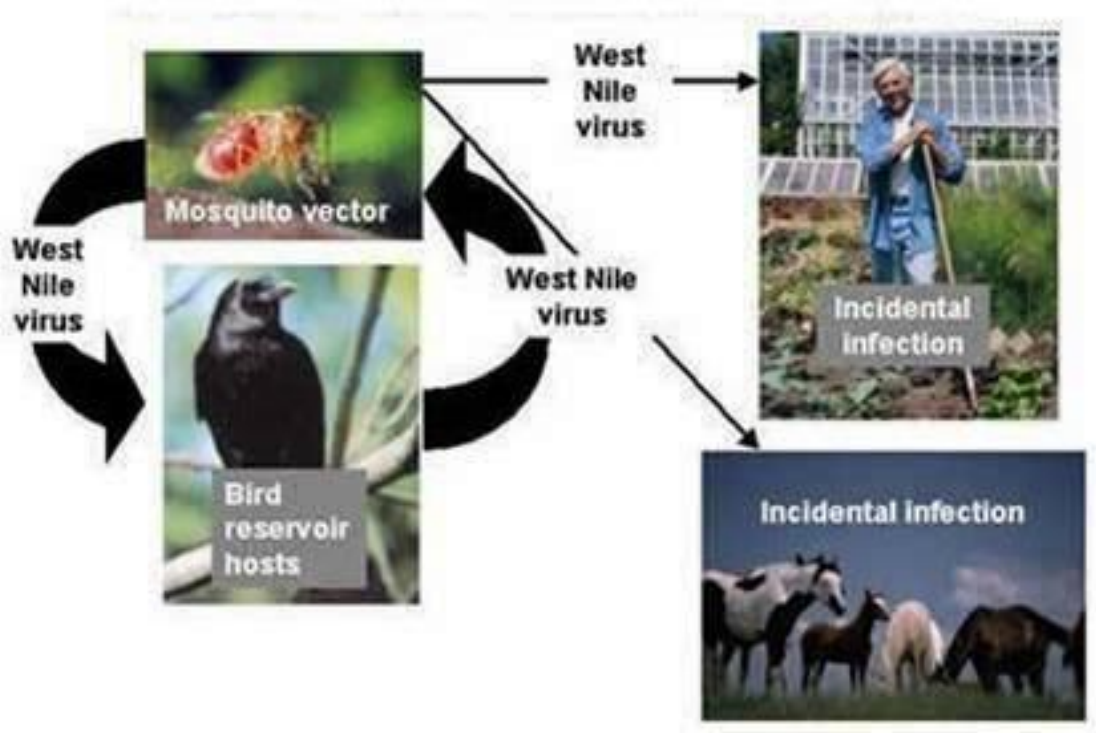
Figure 3.7: Transmission of Dengue virus



3.3.3 Transmission of West Nile Virus

The arboviral like WNV are maintained in complex life cycles involving a non-human primary vertebrate host, usually birds, and a primary arthropod vector. Transmission occurs between susceptible vertebrate hosts by Clulicids (blood feeding arthropod mosquitoes), Phelbotomids (sand flies), Ceratopogonids ('no-see-ums'), Loxodids (hard ticks) and Argasids (soft ticks)[52-55]. Humans, domestic and wild mammals can develop clinical illness and die but usually are incidental or "dead end" hosts, meaning that they contribute little, if at all to the spread of the virus. The reservoir for this virus is a bird, and a recent study suggests the Picuris Ground Doves and Shiny Cowbirds both serve as reservoirs, but the former produces 10 times as many infectious mosquitoes than the later. Humans are not the only one vulnerable to this virus. WNV can also infect domestic animals (cats and dogs), horses, and even bats, squirrels, and rabbits (with less frequency).

Figure 3.8: Transmission of West Nile virus



Birds with severe infections suffer high morbidity and mortality rates, but typically develop life-long immunity after exposure and a short viremia. Mosquitoes acquire the virus when they feed on the infected reservoir birds. Many mammals, including humans and horses, are incidental hosts that become infected when fed upon by an infected mosquito. Although mammals do not develop levels of viremia sufficient to infect mosquitoes, and thus cannot serve as reservoirs, infections in mammals may result in severe, potentially fatal meningoencephalitis. The incubation period of WNV ranges from 2-14 days. Of all people infected with WNV, most will not be aware of the infection and will clear it with no symptoms, 80% of infected individuals did not develop symptoms[42].

3.4 VECTORS

Another way that a disease agent can be introduced is by introduction of an infected vector. Vectors may be either biological vectors that are persistently infected and allow the pathogen to develop and reproduce, or mechanical vectors on which the pathogen resides for a short period of time. Because they are persistently infected, biological vectors are more likely to introduce exotic disease agents of animals to new areas than are mechanical vectors.

3.4.1 Vectors for Japanese Encephalitis

The vectors of JEV are *Culex tritaeniorhynchus, Culex vishnui, Culex pseudovishnui, Culex gelidus, Culex fuscocephala, Culex quinquefasciatus, Culex pipienspallens, Culex bitaeniorhynchus, Culex annulirostris, Aedes togoi, Ae. japonicus, Ae. desvexansnipponii, Anopheles annularis and An. vagus. Culex tritaeniorhynchus* is in the *tritaeniorhynchus* complex, breeds in rice fields, ground pools in vast areas. Two types of mating behavior, eurygamy and moderate stenogamy were detected. In the case of the eurygamy type, the mosquitoes were from Southern Thailand and hilly areas near Kanchanaburi, Thailand[56].

Female mosquitoes are usually dark in color, the cibarial armature has rod teeth and the posterior end of the cibarial armature is bowl shaped with a typical rim. The rim of the bowl is everted. The moderate stenogamy type were mosquitoes from

the plain areas such as Bangkok, Ayutthaya, SuphanBuri and Saraburi. The posterior end of the cibarial armature is bowl shaped with a stout rim. The larvae were characteristic in their siphon index, antennal index, hair of prothoracic segment and comb scale number and arrangement. *Cx. tritaeniorhynchus summorosus* from Japan, Los Banos and Luzon, Philippines, differed from *Cx. tritaeniorhynchus* in that on the lateral plate of the phallosome tritaeniorhynchus teeth are somewhat weakly developed and only gently curved whereas in tritaeniorhynchus summorosus they are strongly developed, considerably longer, and sharply recurved[56].

3.4.2 Vector for Dengue virus

The most common epidemic vector of Dengue in the world is the Aedes aegypti mosquito. It can be identified by the white bands or scale patterns on its legs and thorax. Dengue is transmitted by an infected female mosquito. *Aedes aegypti*is primarily a day time feeder and mainly bites in the morning or late in the afternoon in covered areas. Therefore, this mosquito does not tend to bite at the beach on a sunny day. It is also not usually found in tropical forests or mangroves, except in Africa. The *Aedes aegypti* female prefers to lay its eggs in artificial, rather than natural containers that have fairly clean water and are located around human habitation[57]. Aedes aegypti currently distributed in urban areas throughout the tropical regions of Africa, Asia, Australia, South pacific, America, the origin of the species is considered to be Africa[58].

3.4.3 Vector for West Nile virus

Birds act as both carriers and amplifying hosts of WN virus in nature. Ornithophilic mosquitoes belonging mainly to *Culex* species act as vectors for transmission of infection from viremic birds to a large spectrum of vertebrate hosts. *Culex univittatus* complex (South Africa, Israel), *Culex modestus* (France), *Culex vishnui* complex (India and Pakistan), *Culex pipens* (Romania, USA) acts as major vectors of WN virus. There is no evidence to suggest person-to-person/animal, or animal to animal/person transmission. The virus multiplies in the mosquito vector and after an extrinsic incubation period of about 2 weeks, the vector becomes infective for active transmission to a susceptible host. Hibernating mosquitoes can

carry the virus[59,60] and vertical transmission of the virus from infected female to her progeny has been reported[61]. Migratory birds play a major role in the WN virus dissemination. However, virus dissemination through infected mosquitoes or by illegally imported infected pet birds should also be considered a possibility.

The West Nile virus maintains itself in nature by cycling between mosquitoes and certain species of birds. A mosquito (the vector) bites an uninfected bird (the host), the virus amplifies within the bird, and an uninfected mosquito bites the bird and is in turn infected. Other species such as humans and horses are incidental infections, as they are not the mosquitoes preferred blood meal source. The virus does not amplify within these species and they are known as dead-end hosts.

3.5 CLINICAL FEATURES

3.5.1 Clinical features of Japanese Encephalitis virus

Most JEV infections in humans do not result in apparent illness. The epidemiological data an asymptomatic and symptomatic infection are limited and may vary in different regions. The estimated ratio of symptomatic to asymptomatic infection varies from 1 in 25 to 1 in 1000[62].

The incubation period of JEV is 5–15 days. The clinical syndrome varies from a nonspecific febrile illness to aseptic meningitis to severe encephalitis. Most JE patients are associated with acute short-lived illness followed by prolonged convalescence. Following 2–4 days of nonspecific illness, the patient develops headache, fever and rigor. The gastrointestinal symptoms include nausea, anorexia, vomiting and diffuse abdominal pain which improve in a few days from the date of illness.

During the acute stage of encephalitis, seizures have been reported by various investigators and the frequency ranges from 6.7 to 67.2%[63-67]. The seizures may be focal or secondary generalized and rarely may be associated with status epilepticus. Seizures are more common in children compared to adults.

JE is actually an encephalomyelitis, patients with JE may manifest with variable focal lower motor neuron signs, which may be as subtle as focal reflex loss or as severe as flaccid quadriplegia followed by wasting. Acute flaccid weakness in JE has been reported in 5–20% patients[68,69]. JEV is transmitted through a zoonotic cycle between mosquitoes, pigs and water birds. Human get accidentally infected when bitten by an infected mosquito and are a dead end host.

3.5.2 Clinical features of Dengue virus

The incubation period is 3-14 days (average, 4-7 days) symptoms that begin more than two weeks after a person departs from an endemic area are probably not due to Dengue. Many patients experience a prodrome of chills, erythematous mottling of the skin, and facial flushing (a sensitive and specific indicator of Dengue fever). The prodrome may last for 2-3 days. Children younger than 15 years usually have a nonspecific febrile syndrome, which may be accompanied by a maculopapular rash[70].

Classic Dengue fever begins with sudden onset of fever, chills, and severe (termed break bone) aching of the head, back, and extremities, as well as other symptoms. The fever lasts 2-7 days and may reach 41°C. Fever that lasts longer than 10 days is probably not due to Dengue. Rash in Dengue fever is a maculopapular or macular confluent rash over the face, thorax, and flexor are surfaces, with islands of skin sparing. The rash typically begins on day 3 and persists 2-3 days. Fever typically abates with the cessation of viraemia[70].

The convalescent phase may last for 2 weeks. Patients are at risk for development of Dengue hemorrhagic fever or Dengue shock syndrome at approximately the time of effervescence. Abdominal pain in conjunction with restlessness, change in mental status, hypothermia, and a drop in the platelet count presages the development of Dengue hemorrhagic fever.

3.5.3 Clinical features of West Nile virus

The typical case of West Nile virus were characterized by fever, headache, generalized myalgia, vomiting, diarrhea, and anorexia[71]. The course of fever may be biphasic. Rash occurs in about half of cases with onset either during the febrile phase or at the end of it. The rash is roseolar or maculopapular, nonirritating, and involves the chest, back, and upper extremities. Rash may persist for upto one week and resolves without any desquamation. Generalized lymphadenopathy is a common finding. Pharingitis and gastrointestinal symptoms may occur. Hepatitis, Pancreatitis, myocarditis, cardiac dysrhythmia, rhabdomyolysis, orchitis, uveitis vitreous, optic neuritis, and chorioretinitis have been reported[72-75]. In the Central African Republic, WNV has been responsible for cases of hepatitis, including fatal disease resembling Yellow Fever (YF)[75].

The risk of severe neurologic disease is higher among patients older than 50 years of age and among organ transplant recipients who are immunocompromised[54]. Approximately 50% of persons with neuroinvasive disease will have persistent sequelae 12 months after infection. CDC reports that when the central nervous system (CNS) is affected clinical syndromes ranging from febrile headache to aseptic meningitis to encephalitis may occur, and these are usually indistinguishable from similar syndromes caused by other viruses. About 60 - 75% of people with neuroinvasive WNV infection reportedly have encephalitis or meningoencephalitis, which is characterized by altered mental status or focal neurologic findings. About 25 to 35% of people with neuroinvasive WNV infection reportedly have meningitis without evidence of 12 encephalitis. WN meningitis usually involves fever, headache, and stiff neck. Pleocytosis is present. Changes in consciousness are not usually seen and are mild when present.

3.6 PATHOGENESIS

3.6.1 Pathogenesis of Japanese Encephalitis virus

Crossing the blood–brain barrier is an important factor in the increased pathogenesis and clinical outcome of the neurotropic viral infection. After entering the body through a mosquito bite, the virus reaches the central nervous system

(CNS) via leukocytes (probably T lymphocytes), where JEV virions then bind to the endothelial surface of the CNS and are internalized by endocytosis. Macrophage and axonal transport may play a critical role in JEV pathogenesis. However, convincing evidence is still lacking[76-80].

JE typically develops in patients after an incubation period of 5–15 days. It is possible that during this time, the virus resides and multiplies within host leukocytes, which act as carriers to the CNS. T lymphocytes and IgM play a major role in the recovery and clearance of the virus after infection[78].

A plausible therapy of clearing the virus load while in its incubation period in peripheral lymphatic tissues and spleen may actually prevent JEV pathogenesis. The molecular pathogenesis of JEV infection is still unclear. Reports suggest that JEV infection affects neuronal progenitor cells (NPCs) in the sub ventricular zone and severely compromises their ability to proliferate. JEV infection does not result in the death of resilient NPCs, but the cycling ability of these cells is suppressed. This arrested growth and proliferation of NPCs might be the cause of neurological consequences in children infected by JEV[81-85]. Also, there are reports that JE can be transmitted transplacental by which means the virus could affect the normal neuronal development of the fetus[86].

3.6.2 Pathogenesis of Dengue virus

After inoculation the virus replicates in nearby lymph node cells. Viremia follows and reticuloendothelial cells in skin and other tissues grow to be infected. Local inflammatory changes happen around tiny vessels in the skin[87]. Dengue hemorrhagic fever occurs in kids who have previously been exposed to infection with a diverse serotype of the virus, or who have acquired antibody passively from their mother.

The incubation period is about 7 days prior to the onset of high fever, headache, eye pains, backache and chills. Limb pain is often severe in Dengue and this gives rise to its common name, break bone fever. A blanching erythematous macular rash may appear on the third or fourth day of the illness. Lymphadenopathy

could be present. Encephalopathy, cardiomyopathy and liver damage also occur. Leucopenia is usual in the peripheral blood. In Dengue hemorrhagic fever the patient is far more ill, and blood pressure falls as a result of transudation of fluid from the vascular compartment[60,88-91].

This fluid can accumulate in the abdominal cavity or in the pleural spaces. There might be spontaneous bleeding into the skin and at other sites. The loss of circulating blood volume causes shock, with low blood pressure, rapid pulse, restlessness and abdominal discomfort (the Dengue shock syndrome), which can have a mortality of 50% if untreated[87,70].

Molecular studies have demonstrated that Dengue viruses vary genetically in nature unfortunately, phenotypic changes that have been observed in the field have not yet been associated with genetic changes in the virus. The viral factors play a significant role in the pathogenesis of severe Dengue disease[92-97].

3.6.3 Pathogenesis of West Nile virus

The mechanism by which WNV and other neurotropic viruses cause the blood-brain barrier (BBB) remain largely unknown, although tumor necrosis factor alpha (TNF-α)-mediated changes in endothelial cell permeability may facilitate central nervous system (CNS) entry. It is likely that WNV infects the CNS at least in part via hematogeneous spread, as an increased viral burden in serum correlates with earlier viral entry into the brain[98-100].

Additional mechanisms may contribute to WNV CNS infection, including infection or passive transport through the endothelium or choroid plexus epithelial cells[102] infection of olfactory neurons and spread to the olfactory bulb[103] direct axonal retrograde transport from infected peripheral neurons[104,105]. Although the precise mechanisms of WNV CNS entry in humans require additional study, changes in cytokine levels that may modulate BBB permeability and infection of blood monocytes and choroid plexus cells have been documented in animal models[101,106,99].

Infection or passive transport trough the endothelium or choroid plexus epithelial cells. Infection of olfactory neurons and spread to the olfactory bulb. A "Trojan horse" mechanism in which the virus is transported by infects immune cells that traffic to the CNS[101].

Direct axonal retrograde transport from infected peripheral neurons. Although the precise mechanisms of WNV CNS entry in humans require additional study, changes in cytokine levels that may modulate BBB permeability and infection of blood monocyte and choroid plexus cells have been documented in animal models[101,104].

3.7 EPIDEMIOLOGY

3.7.1 Epidemiology of Japanese Encephalitis virus

The epidemic of viral encephalitis was reported from July through November 2005 in Gorakhpur, Uttar Pradesh, India. It was the longest and most severe epidemic in 3 decades; 5,737 persons were affected in 7 districts of eastern Uttar Pradesh, and 1,344 persons died[107]. Japanese Encephalitis virus (JEV) is the most common cause of childhood viral encephalitis in the world, it causes an estimated 50,000 cases and 10,000 deaths annually[108,109]. JEV is endemic in the Gorakhpur and Basti divisions of Eastern Uttar Pradesh. The geographic features of this region are conducive for the spread of JEV; an abundance of rice fields and a bowl-shaped landscape allow water to collect in pools. Heavy rains saturated the ground in 2005, which caused ideal breeding conditions for mosquitoes that transmit the virus from pigs to humans.

Acute Encephalitas Syndrome (AES) occur regularly in several parts of India. Japanese Encephalitis virus (JEV) has been the major and consistent cause of these outbreaks in the Gorakhpur region of Uttar Pradesh state, accounting for 10-15% of total AES cases annually[110,111,112]. In India, vaccinations against Japanese Encephalitis (JE) are administered in areas where the disease is hyperendemic, including Gorakhpur, and AES cases are regularly investigated to clarify the effects of vaccination. Currently, >2,000 patients with AES are admitted each year to Baba Raghav Das Medical College, Gorakhpur.

JEV is classified into 5 genotypes. Genotype III (GIII) is widely distributed in Asian countries, including Japan, South Korea, and the people's Republic of China, Taiwan, Vietnam, the Philippines, India, Nepal, and Sri Lanka[113]. However, during the past decade, JEV GI has been introduced into South Korea, Thailand, and China and has replaced the GIII strains that have been circulating in Japan and Vietnam during the mid-1990[114]. Until 2007, all known JEV strains isolated in India[115].

Japanese Encephalitis is a seasonal disease, with most cases occurring in temperate areas from June to September. Further South, in subtropical areas, JEV transmission begins as early as March and extends until October. Transmission may occur all year in some tropical areas (e.g., Indonesia). Globally, more than 45,000 cases are reported each year, although this is likely an under estimation of the true incidence of the disease[120]. Local incidence rates range from 1-10 cases per 100,000 persons but can reach more than 100 cases per 100,000 persons during outbreaks. The travel associated risk is overall relatively low (1 per 5,000–20,000 per week of travel), but the severity of natural infection and possible complications has been important factors that promote vaccination as a major preventive practice.

Although most human infections are mild or asymptomatic, about 50% of patients who develop encephalitis suffer permanent neurologic defects and 30% of them die due to the disease [116]. In 1973, JE outbreak was first recorded in the districts of Burdwan and Bankura in West Bengal where 700 cases and 300 deaths were reported[117-119].

3.7.2 Epidemiology of Dengue virus

Dengue is the most important arthropod-borne viral disease of public health significance. Compared with nine reporting countries in the 1950s, today the geographic distribution includes more than 100 countries worldwide. Many of these had not reported Dengue for 20 or more years and several have no known history of the disease. The World Health Organization estimates that more than 2.5 billion people are at risk of Dengue infection. First recognized in the 1950s, it has become a leading cause of child mortality in several Asian and South American countries[121]

Most people with Dengue recover without any ongoing problems. The mortality is 1–5% without treatment[68].

In India, the first epidemic of clinical Dengue-like illness was recorded in Madras (now Chennai) in 1780 and the first virologically proved epidemic of Dengue fever (DF) occurred in Calcutta (now Kolkata) and Eastern Coast of India in 1963-1964. During the last 50 years a large number of physicians have treated and described Dengue disease in India, but the scientific studies addressing various problems of Dengue disease have been carried out at limited number of centers[122].

Dengue has been present for centuries. The first recorded symptoms compatible with Dengue were noted in Chinese medical encylopedia in 992 AD, however originally published by the Chin Dynasty centuries earlier(265-420 AD) prior to being formally edited[123]. The disease was referred to as water poison and was associated with flying insects[124]. Epidemics that resembled Dengue, with similar disease course and spread, occurred as early as 1635 and 1699 in the West Indies and Central America[125].

A major epidemic occurred in Philadelphia in 1780 and epidemics then became common in the USA into the early 20th century, the last outbreak occurring in 1945 in New Orleans[125,126]. The viral etiology and the transmission by mosquitoes were only finally determined in the 20th century. The origin of the primary mosquito vector, *A. aegypti*, is debated to be from either Africa or Asia[127,125].

Epidemics were spaced by 10-40 year intervals due to this shipping mode of transport[126,123,128]. Expansion of the disease heightened during World War II (WWII), when troops began to disperse inland and utilize modern transportation within and between countries, thus epidemic Dengue became more far-reaching[126]. By the end of the war, transportation and rapid urbanization led to increased transmission of Dengue and hyperendemicity (multiple serotypes present) in most South East Asian countries, with subsequent emergence of the severe forms of Dengue[129,130]. Global Dengue incidence has increased precipitously over the last five decades and severe Dengue cases have also expanded[129,131,132].

Transmission of Dengue is now present in every World Health Organization (WHO) region of the world and more than 125 countries are known to be Dengue endemic. The true impact of Dengue globally is difficult to ascertain due to factors such as inadequate disease surveillance, misdiagnosis, and low levels of reporting. Currently available data likely grossly underestimates the social, economic, and disease burden. Estimates of the global incidence of Dengue infections per year have ranged between 50 million and 200 million; however, recent estimates using cartographic approaches suggest this number is closer to almost 400 million[133].

3.7.3 Epidemiology of West Nile virus

The first reported case of West Nile virus came from the West Nile District of Uganda (1937)[134]. As of 2004, the virus has been detected throughout the entire United States. The peak incidence in North America falls between August and September. To date, WNV has been seen in Europe, Africa, parts of Asia, the Middle East, and North America[135,136].

WNV specific neutralizing antibodies have been detected in America, Borneo, China, Georgia, Iraq, Uganda, Kenya, Lebanon, Malaysia, Phillippines, Srilanka, Syria, Thailand, Tunisia, Turkey Belgian Congo and Sudan[135,137]. Recently the virus has been recognized in New York, America[138].

WNV is an emerging virus infection of the globe. Several outbreaks in different countries with various range of severity has been reported earlier[136-140]. The first known outbreak of WNV in the northern United states was observed during late August 1995 and due to the widespread virus activity, North Eastern USA is becoming endemic to WNV[141,142,55]. In human, clinically WNV appears as a mild, self limited, non-fatal, febrile illness rarely leading to encephalitis. However, myocarditis, a rare non-neurological complication[143] and pancreatitis WNV infection[144] and also have been reported.

WNV has been isolated from sporadic cases of encephalitis and mosquitoes[145]. Work postulated a hypothesis of a zoogeographical interface of Japanese Encephalitis and West Nile virus. The hypothesis proposed the

intermingling distribution of JEV and WNV at the south Indian peninsular region. From the available data, it is evident that different viruses may predominate in different years since in south Arcot district of Tamil Nadu,[146] observed a higher prevalence of neutralizing antibodies to WNV than JEV during a post encephalitis outbreak survey in 1982, whereas in the same area, during 1989 and 1990 low prevalence of WNV. The increase in frequency of outbreaks, severe disease in humans and horses and high mortality rates in birds have emerged as the disturbing trends in the epidemiology of WN fever.

Horses encounter the WNV infection like humans and experience encephalitis. Though the WNV encephalitis in horses is rare, a considerable mortality rate is reported in the case of encephalitis horses. WNV fever in horses has been reported in Egypt[147], France[148], Morocco[149] Italy[150] and in USA, infection in horses has not been documented in India. However in a recent survey, a significant rate of serological evidence against WNV has been noticed among horses in and around Pune city. Extensive studies of WNV infection in horses in India needs to be carried out.

3.8 SIGNS AND SYMPTOMS

3.8.1 Signs and symptoms of Japanese Encephalitis virus

Japanese Encephalitis has an incubation period of 5 to 15 days and the vast majority of infections are asymptomatic: only 1 in 250 infections develop into encephalitis[62].

Most JE virus infections are mild (fever and headache) or without apparent symptoms, but approximately 1 in 250 infections results in severe disease characterized by rapid onset of high fever, headache, neck stiffness, disorientation, coma, seizures, spastic paralysis and death. The case-fatality rate can be as high as 30% among those with disease symptoms. Of those who survive, 20–30% suffer permanent intellectual, behavioral or neurological problems such as paralysis, recurrent seizures or the inability to speak[151].

Severe rigors mark the onset of this disease in humans. Fever, headache and malaise are other non-specific symptoms of this disease which may last for a period of between 1 and 6 days. Signs which develop during the acute encephalitic stage include neck rigidity, cachexia, hemiparesis, convulsions and a raised body temperature between 38 and 41°C. Mental retardation developed from this disease usually leads to coma[151].

Mortality of this disease varies but is generally much higher in children. Transplacental spread has been noted. Life long neurological defects such as deafness, emotional ability and hemiparesis may occur in those who have had central nervous system involvement. In known cases some effects also include nausea, headache, and fever, vomiting and sometimes swelling of the testicles[152].

Increased microglia activation following JEV infection has been found to influence the outcome of viral pathogenesis. Microglia are the resident immune cells of the central nervous system (CNS) and have a critical role in host defense against invading microorganisms. Activated microglia secrete cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α), which can cause toxic effects in the brain. Additionally, other soluble factors such as neurotoxins, excitatory neurotransmitters, prostaglandin, reactive oxygen, and nitrogen species are secreted by activated microglia[153].

The severity of the disease in humans can be divided into 3 stages.

- **Prodromal stage**-the onset of illness is usually acute and is heralded by fever, headache and malaise. The duration of this stage is usually 6 days.
- **Acute Encephalitis Stage**-Fever is usually high, 38-41°C the prominent features are fever, neck rigidity convulsions (especially in infants) and altered sensorium progressing on many cases to coma.
- **Sequelae**-More severe infection is marked by quick onset headache, high fever, neck stiffness, stupor, disorientation, spastic paralysis followed by gradual disturbances in speech change is mental status. (National vector borne disease control programme).

3.8.2 Signs and Symptoms of Dengue virus

Dengue is a complex disease with a wide spectrum of clinical presentations, which often goes unrecognized or is misdiagnosed as other fever-causing tropical diseases[154,155]. Following the period of incubation, most patients experience a sudden onset of fever which can remain for 2–7 days and is often accompanied with symptoms such as myalgia, arthralgia, anorexia, sore throat, headaches, and a macular skin rash[156,157]. It is during this period that differentiating Dengue from other febrile diseases proves troublesome[158].

The majority of people experience a self-limiting clinical course, which does not progress to the severe forms of Dengue, Dengue hemorrhagic fever (DHF), or Dengue shock syndrome (DSS). Secondary Dengue infections or particularly virulent viral strains are two factors thought to be associated with increased risk of severity[129,159]. In severe cases, thrombocytopenia and increased vascular permeability can result in hemorrhagic and shock complications. Currently, neither a vaccine nor specific antiviral therapy exists[160,161,162].

The characteristic symptoms of Dengue are sudden-onset fever, headache (typically located behind the eyes), muscle and joint pains, and a rash. The alternative name for Dengue, "break bone fever", comes from the associated muscle and joint pains. The course of infection is divided into three phases: febrile, critical, and recovery[70].

The febrile phase involves high fever, often over 40°C (104°F), and is associated with generalized pain and a headache; this usually lasts two to seven days. Vomiting may also occur. A rash occurs in 50–80% of those with symptoms in the first or second day of symptoms as flushed skin, or later in the course of illness (days 4-7), as a measles like rash. Some petechial (small red spots that do not disappear when the skin is pressed, which are caused by broken capillaries) can appear at this point, as may some mild bleeding from the mucous membranes of the mouth and nose. The fever itself is classically biphasic in nature, breaking and then returning for one or two days, although there is wide variation in how often this pattern actually happens[124,157].

In some people, the disease proceeds to a critical phase around the time fever resolves[160] and typically lasts one to two days. During this phase there may be significant fluid accumulation in the chest and abdominal cavity due to increased capillary permeability and leakage. This leads to depletion of fluid from the circulation and decreased blood supply to vital organs. During this phase, organ dysfunction and severe bleeding, typically from the gastro-intestinal tract, may occur[68]. Shock (Dengue shock syndrome) and haemorrhage (Dengue hemorrhagic fever) occur in less than 5% of all cases of Dengue, however those who have previously been infected with other serotypes of Dengue virus (secondary infection) are at an increased risk. This critical phase, while rare, occurs relatively more commonly in children and young adults.

The recovery phase occurs next, with resumption of the leaked fluid into the bloodstream[108]. This usually lasts two to three days. The improvement is often striking, but there may be severe itching and a slow heart rate. Another rash may occur with either a maculopapular or a vasculitic appearance, which is followed by peeling of the skin. During this stage, an overload state may occur, if it affects the brain, it may cause a reduced level of consciousness or seizures. A feeling of fatigue may last for weeks in adults.

3.8.3 Signs and symptoms of West Nile virus

The incubation period for WNV the amount of time from infection to symptom onset is typically from between 2 and 15 days. Headache can be a prominent symptom of WNV fever, meningitis, encephalitis, meningoencephalitis and it may or may not be present in poliomyelitis-like syndrome thus headache is not a useful indicator of neuroinvasive disease[54].

West Nile fever (WNF), which occurs in 20 percent of cases, is a febrile syndrome that causes flu-like symptoms. Most characterizations of WNF generally describe it as a mild, acute syndrome lasting 3 to 6 days after symptom onset. Systematic follow-up studies of patients with WNF have not been done, so this information is largely anecdotal. In addition to a high fever, headache, chills, excessive sweating, weakness, fatigue, swollen lymph nodes, drowsiness, pain in the

joints and flu-like symptoms[74]. Gastrointestinal symptoms which may occur include nausea, vomiting, loss of appetite, and diarrhea. Less than one-third of patients develop a rash. West Nile neuroinvasive disease (WNND), which occurs in less than 1 percent of cases, is when the virus infects the central nervous system resulting in meningitis, encephalitis, meningoencephalitis or a poliomyelitis-like syndrom[54].

West Nile virus encephalitis (WNE) is the most common neuroinvasive manifestation of WNND. WNE presents with similar symptoms to other viral encephalitis with fever, headaches, and altered mental status. A prominent finding in WNE is muscular weakness (30 to 50% of patients with encephalitis), often with lower motor neuron symptoms, flaccid paralysis, and hyporeflexia with no sensory abnormalities . WNV is now the most common cause of epidemic viral encephalitis in the United States and it will likely remain an important cause of neurological disease for the foreseeable future.

3.9 RECENT OUTBREAKS

3.9.1 Recent Outbreak of Japanese Encephalitis in India

In India, the first human case was reported from North Arcot district of Tamil Nadu in 1955. Until 1973, the disease was confined to Southern parts of India, with low prevalence, subsequently the disease spread to various other parts of India. In 1978, suspected outbreaks of JE were reported from 18 states and 24 states and Union Territories have reported suspected JE cases till recent past[163].

Japanese Encephalitis is severe disease and its epidemics are reported from many parts of India[164]. Initial cases of JEV were reported in the 1950s in India[165]. The majority of the JEV cases during the year 1978, epidemic of JEV from eastern Uttar Pradesh (Gorakhpur and adjoining areas), which is the Terai area. Uttar Pradesh (a Northern State of India) and is surrounded by Urraranchal in the North East, Haryana and Himachal Pradesh in the North, Delhi and Rajasthan in the West, Madhya Pradesh in the South West, Chattisgarh in the South, Bihar in the South East and Nepal along the East[164,166].

During 20th century extensive outbreaks of encephalitis was recognized in North Eastern India with 7463 cases reported. In the North region, Uttar Pradesh experienced its first epidemic in 1979 and the disease has continued there with 1716-3894 cases per year. The outbreak of encephalitis have been reported from different parts of India, predominantly in the rural areas of Bihar, Uttar Pradesh, Assam, Manipur, Andhra Pradesh, Karnataka, Madhya Pradesh, Maharashtra, Tamil Nadu, Haryana, Kerala, West Bengal, Orissa and union territories of Goa and Pondicherry[167]. Occurrence of Epidemic of Japanese Encephalitis in Andhra Pradesh, during 1999, caused 178 deaths out of 873 peoples were affected[168]. In Northern states, the disease was reported between 1997 and 1981.

3.9.2 Recent Outbreak of Dengue in India

Dengue infection is endemic in many parts of India. In India, the first epidemic of clinical Dengue-like illness was recorded in Madras (now Chennai) in 1780. The first virologically proven epidemic of DF in India occurred in 1963-1964[169-171].

DHF was occurring in the adjoining countries but it was absent in India for unknown reasons as all the risk factors were present. The DHF started simmering in various parts of India since 1988[172-174]. The first major wide spread epidemics of DHF/DSS occurred in India in 1996 involving areas around Delhi[175] and Lucknow[176] and then it spread to all over the country[177].

The very first report of existence of Dengue fevers in India was way back in 1946[178]. An initial epidemic of Dengue fever was reported on the Eastern Coast of India[171,18,179-187] it spread northwards and reached Delhi in 1967[183] and Kanpur in 1968[184,185]. Simultaneously it also involved the Southern part of the country[186,187] and gradually the whole country was involved with wide spread epidemics followed by endemic/hyperendemic prevalence of all the four serotypes of DV.

DENV-2 was isolated during the epidemics of Dengue in urban and rural areas of Gujarat State during 1988 and 1989[188]. Outbreaks of Dengue occurred in

Rajasthan by DENV- 1 and DENV-3[189], DENV-3[190], Madhya Pradesh by DENV-3[191], Gujarat by DENV-2[188] and in Haryana by DENV-2[192]. DENV-2 was the predominant serotype circulating in Northern India, including Delhi, Lucknow and Gwalior[175,176,193] while DENV-1 was isolated during the 1997 epidemic at Delhi[194]. The phylogenetic analysis by the Molecular Evolutionary Genetics Analysis programme suggests that the 1996 Delhi isolates of DENV-2 were genotype IV. The 1967 isolate was similar to a 1957 isolate of DENV-2, from India, and was classified as genotype V. This study indicates that earlier DENV-2 strains of genotype V have been replaced by genotype IV[195].

3.9.3 Recent Outbreak of West Nile Virus in India

Though West Nile virus is reason for the encephalitis cases it can be said that in India the prevalence is very less compared to the outbreaks happened in U.S, Africa and European countries. The ratio of West Nile cases causing AES will be 2-3% only. But when the epidemic outbreak happen the percentage of WNV causing AES might shoot up. Epidemiology of WN infection is not well known in India. Serological survey during JE epidemics and in areas endemic to JE shows that the virus is prevalent in India[142].

Fatal cases were seen in children[196] unlike in the older age groups in the recent outbreaks in other countries. Despite the presence of mosquito vectors in abundance, and potentially neurovirulent strains of the virus, there have been no serious epidemics due to WNV in India comparable to JE outbreaks. Reasons for this situation are not clear, but presence of other flaviviruses in India could be an important factor for limiting the spread of WN or reducing its severity.

An outbreak of acute encephalitis syndrome (AES) was reported in Kerala in India in May 2011. Serum neutralization assay result revealed that 32 of 42 (76.19%) sera were positive for WNV neutralization antibodies[197].

3.10 LAB DIAGNOSIS

1. Attempts to isolate virus from the blood of patients with flavivirus encephalitis are usually unsuccessful because viremia is transient and titres are low.

2. Virus is occasionally isolated from the cerebrospinal fluid of patients who do not yet have antibody.

3. Viral ribonucleic acid occasionally may be detected in the cerebrospinal fluid by the reverse transcription polymerase chain reaction (PCR)[198] for West Nile virus, real-time PCR has proved more useful.

4. However, the accepted standard for diagnosing flavivirus encephalitis is the IgM capture enzyme-linked Immunosorbent assay (ELISA). This assay will often detect antibody on a single cerebrospinal fluid or serum sample.

3.10.1 Culture

Culturing the virus in susceptible cell cultures, such as rabbit kidney (RK-13) and African green monkey kidney (Vero) cells, or embryonating chicken eggs. Intracerebral inoculations of newborn mice are less likely to yield virus isolates from mammalian tissues than cell culture methods. More than one cell culture passage may be required to observe cytopathic effect (CPE). Virus culture is the gold standard, but it is rarely positive except in autopsy material, generally from the brain and other solid organs.

3.10.2 Virus isolation

Virus has rarely been cultured from serum, CSF and other tissues. Propagation of virus requires use of live cell cultures or suckling mice, and must be performed in a biosafety level 3 laboratory. Culture is insensitive compared with other techniques and is time-consuming, thus remains primarily a research tool.

3.10.3 Complement fixation (CF) test

CF assays capitalize on the ability of antigen-antibody complexes to trigger the complement cascade, and the sensitivity of antibody-coated erythrocytes to complement-mediated lysis[199]. If patient serum contains complement-fixing antibodies recognizing the antigen in question, exogenous (guinea pig) complement added to the system will be consumed, and thus unavailable to lyse the indicator

cells (antibody-coated erythrocytes) added to the system at a later time point. Thus, the absence of hemolysis (i.e., a visible red cell pellet topped by clear liquid) indicates the presence of antibodies to the antigen in question. Serial dilutions of a given specimen are tested, and the CF titre is defined as the highest dilution showing 50% hemolysis. CF antibodies induced by flavivirus infections typically appear within 2 weeks after onset, and their levels begin to decrease about 2 months later, reaching baseline levels in 1–2 years[200].

Some flavivirus infected individuals do not produce complement-fixing antibodies; approximately 20% of patients infected with SLEV during an outbreak remained negative for CF antibodies for up to 8 weeks[201]. Other problems reflect the highly complex nature of the assay; it is labor intensive, requires highly trained personnel, and demands strict quality control of reagents. Although historically important, CF testing for WNV antibodies is rarely performed today; other assays discussed below have proven more sensitive and much less labor intensive.

3.10.4 Haemagglutination inhibition (HAI) assay

The HI assay capitalizes on the finding that flavivirus envelope proteins (haemagglutinins) bind to and agglutinate avian erythrocytes, and that antibodies from infected individuals block this agglutination[118]. Acetone-extracted serum is first absorbed with goose erythrocytes, followed by serial dilution; a defined amount of antigen prepared from suckling mouse brains is then added to each serum dilution. After an overnight incubation in the refrigerator, goose erythrocytes are added to each serum-antigen mixture; after an hour at room temperature, agglutination is scored. A thin mat of erythrocytes indicates agglutination, and thus the absence of serum antibodies capable of inhibiting agglutination; in contrast, a pellet of erythrocytes indicates agglutination inhibition, and thus the presence of antibodies capable of blocking the interaction between antigen and the goose erythrocytes. The HI titre is defined as the highest serum dilution completely inhibiting agglutination.

3.10.5 Plaque reduction neutralization test (PRNT)

Although developed many years ago, the PRNT still serves as the gold standard for measuring specific antibodies, due to its exquisite specificity[202]. Multiple dilutions of patient serum are incubated with a live virus preparation containing a defined number of infective units. The serum-virus mixtures are then added to culture wells containing a confluent monolayer of Vero cells, which are susceptible to flavivirus infection. After culturing for a defined number of days, the number of plaques (visible areas of nonviable cells killed as a result of viral infection) is determined.

Flavivirus specific antibodies in the serum bind to envelope proteins and neutralize the ability of the virus to infect Vero cells, as evidenced by a reduction in the number of plaques. The highest serum dilution reducing plaque formation by a given level (typically 80-90% reduction in plaque count) is defined as the endpoint titre. It is very useful in survey and confirmation of virus isolates. It aids in discriminating between the different flaviviruses because interpreting antibody levels is difficult, because flavivirus shows the cross reactivity with each other due to antigenic sharing among members of viridae. PRNT is the most specific test for the arthropod-borne flaviviruses, which can be used to help distinguish false-positive results in an IgM antibody- capture enzyme-linked immunosorbent assay or other assays (for example, indirect immunofluorescence and haemagglutination inhibition).

3.10.6 Enzyme Linked Immunosorbent Assay (ELISA)

The most efficient diagnostic method is detection of IgM antibody to WNV in serum collected within 8 to 14 days of illness onset or CSF collected within 8 days of illness onset for IgM antibody-capture, enzyme-linked immunosorbent assay (MAC-ELISA), which is routinely used for the diagnosis of acute infection in humans[203]. Most patients with WNND have detectable antibody in CSF and serum at the onset of neurological symptoms[204]. By the 8th day of illness, a large majority of infected persons will have detectable serum IgM antibody to flavivirus infection. In most cases it will be detectable for at least 1-2 months after illness onset, in some

cases it will reach undetectable levels prior to 1 month after illness onset or it will be detectable for 12 months or longer. By 3 weeks of post infection (often earlier), virtually all infected persons demonstrate long-lived serum IgG antibody to WNV.

The most effective means of diagnosing flavivirus infection is antibody detection. Nearly all AES infected individuals are seropositive for flavivirus infection IgM within 14 days of the onset of symptoms. Essentially all patients with neurologic involvement have detectable IgM result for CSF reflects inthrathecal antibody synthesis, and is thus diagnostic of central nervous system infection.

3.10.7 Reverse transcription Polymerase chain reaction (RT-PCR)

PCR is a sensitive technique for identifying flavivirus genetic material in both serum and cerebrospinal fluid (CSF) as samples collected on days of onset below 5 days. RT-PCR protocol remains a useful tool for surveillance studies and evolutionary research owing to the possibility of obtaining epidemiological data through the sequence data. At present, an extensive number of Taq Man and SYBR-Green RT-PCR commercial kits are available that are helpful for the standardization of robust and reliable real-time protocols, providing a good profile of sensitivity and specificity without needing extensive standardization.

3.10.8 Indirect immunoflourescence assay (IFA)

Confirmation of flavivirus isolates is achieved by indirect fluorescent antibody staining of infected cultures. The solid phase for the WNV antibody IFA is a glass slide well coated with fixed WNV-infected cells; diluted serum or CSF is added to the well, and after incubation and washing, fluorescein-labeled anti-human IgG or anti-human IgM is added. After another incubation and wash step, a cover slip is applied, and the slide is examined using a fluorescent microscope. A clearly visible cytoplasmic fluorescent pattern is interpreted as positive[205,206].

Slides for performing IFA are commercially available from Pan-Bio (Columbia, MD). Two technical issues regarding IFA performance are worth noting. First, the cell preparation bound to the glass slide well is typically a mixture of virus

infected and uninfected cells; thus, only some of the cells (typically 30%–50%) exhibit fluorescence when treated with an antibody-positive sample. Detection of fluorescence in all the cells is indicative of nonspecific staining, and thus cannot be interpreted as positive. Second, serum specimens for IgM determination should be diluted in sample buffer containing anti-human IgG, in order to remove the IgG in the sample. This treatment prevents false negative IgM results due to competition between IgG and IgM for available antigen binding sites; it also eliminates false positive IgM results caused by attachment of IgM rheumatoid factor to bound WNV specific IgG[205,207]. IFA offer some notable advantages over CF, HI and PRNT. The complex antigen preparation procedures of CF and HI are not required and in contrast to the PRNT, the risk of accidental infection of the person performing the assay is eliminated by fixation of infected cells.

Since IFA can be performed in a single day, results are available much sooner compared to the other assays mentioned and also, the sample volume required for IFA is lower, making evaluation of CSF more practical[205]. The major advantage, however, is the ability to separately measure IgM and IgG antibodies by IFA, as opposed to measurement of total antibodies by the previously described methods. This information can be used to approximate the time since infection, particularly in situations such as seroprevalence studies, where a single specimen is tested and patient histories are not available.

3.10.9 Micro sphere immunoassay (MIA)

All screening assays for WNV antibodies share the problem of cross reactivity with other flavivirus infections. Specific identification of the infecting flavivirus is achieved by concurrent testing for antibodies to flaviviruses found in a given geographic area. MIA may provide the means to simultaneously assess antibody levels to multiple flaviviruses rapidly at a reasonable cost.

The most widely used MIA is the system developed by Luminex Corporation (Austin, TX). This system uses multiple polystyrene bead sets; each bead set contains distinctive proportions of red and orange fluorescent dyes that yield a signature fluorescent pattern when analyzed by a modified flow cytometer[205]. In its

most basic format for detecting IgG to different antigens, each of the distinctively fluorescent bead sets can be covalently linked to a different antigen. These bead sets, each with a different antigen target, can then be mixed together in a single reaction well. Serum is added, and antibodies recognizing the antigens in the mixture bind to the antigens. Goat anti-human IgG conjugated to a fluorescent reagent (phycoerythrin) is then added as a reporter antibody, and binds to any captured IgG. The bead mixture is then simultaneously analyzed for the bead sets' signature fluorescent patterns and the reporter antibody. The reporter fluorescence intensity is directly proportional to the amount of antigen-specific IgG bound to a given bead set. Thus, antibodies to multiple antigens can be measured in a single reaction well[205]. MIA offers greater sensitivity than ELISA systems due to (a) a broad dynamic range, (b) more surface area resulting in more available antigenic epitopes, and (c) the superior fluorescent characteristics of phycoerythrin. Less specimen volume is required for MIA because multiple assays are performed in a single reaction well, and the high precision of the assay eliminates the need for replicate testing. Finally, MIA is cost-effective, particularly from the standpoint of tests performed per microgram of antigen.

3.10.10 Nucleic acid amplification tests (NAAT)

Nucleic acid amplification testing (NAAT) plays an important role in avian and mosquito surveillance programmes[208]. NAAT testing has been applied to diagnosis of human WNV disease with varying results. Early studies suggested a limited rule for NAAT based on the short duration of viraemia[209]. A more recent study performed during a large community outbreak of WNV infection in Canada found that 45% of plasma samples from patents with WNV infection had detectable virus[210].

In this study, the primary determinant of a positive PCR was duration of symptoms. Plasma NAAT was positive among 56% of patients presenting within eight days of symptom onset compared with 4% of those presenting more than a week into clinical illness. The plasma viral load ranged from 50 copies to 1.4x105 copies/ml (mean 7.5 x103 copies/Ml), with no association between the quantity of

virus detected and the duration of symptoms and the presence of CNS involvement. Patients with WNND are much less likely to have detectable virus compared with those with WNF, perhaps reflecting the delayed onset of neurological symptoms. In the Canadian study cited above, 36% of patients with WNF had detectable virus in plasma compared with only 9.5% of those with WNND[210].

PCR is a sensitive technique for identifying WNV in brain tissue, but is of limited use in detecting virus in spinal fluid. PCR is used in the diagnosis of WNV infections in humans, although it has limited usefulness because of the transient and low viremias. With PCR, WNV genetic material can be detected in CSF in up to 50% of patients who present with acute West Nile meningoencephalitis.

One study evaluating CSF PCR among patients with serologically confirmed WNND identified virus in 57% of samples using real-time PCR, and in 0% of specimens using conventional PCR[211]. The low yield of CSF PCR for diagnosis of WNV has been replicated by other investigators[212]. While NAAT testing is not routinely recommended in immunocompetent hosts, molecular amplification tests may be diagnostically indicated for identification of WNV infection in immunocompromised patients, a population at high risk of central nervous system involvement and adverse outcomes.

Case reports have documented positive NAAT in transplant patients and those with hematological malignancies. In this population viraemia may persist for a prolonged period of time, congruent with the delay in detectable neutralizing antibody[213,214]. Techniques that have been studied include real-time reverse transcription polymerase chain reaction (RT-PCR) and nucleic acid sequence-based amplification (NASBA), both of which are able to detect as few as 50 viral RNA copies/ml[215].

Recently, the RT-PCR method for the detection of virus specific genome has been extensively used by several workers[216,217,218]. To increase the sensitivity and specificity certain modified assays *viz.,* TaqMan assay[216] and nucleic acid sequence based amplification method (NASBA)[219] have been reported. The TaqMan assay has been successfully used for the detection of WNV in human CSF (cerebrospinal fluid)

that was found negative in conventional cell culture technique[216]. The other NASBA method involves the use of three enzymes and a detection system of electrochemiluminescence (ECL). Alternately, molecular beacon probes have also been used as detection system[219].

3.11 PREVENTION OF FLAVIVIRUS INFECTION

Eight flaviviruses cause significant morbidity and mortality around the globe: Japanese Encephalitis (JE), Dengue 1, 2, 3, 4 and West Nile (WN). JEV and WN are zoonosis, with the consequence that vaccines are the only means of protecting humans. The successful YF 17D vaccine, introduced in 1937, produced dramatic reductions in epidemic activity[220].

For many years, only inactivated JE vaccines prepared from infected mouse brains were licensed for use by residents and travellers. Use of this vaccines resulted in an unacceptable level of adverse safety events and it production has been discontinued by major manufactures. Recently, JE vaccine landscape has changed. A safe and efficacious single dose, live attenuated vaccine produced in China has become available in many Asia countries. A new inactivated JE alum adjuvant vaccine is now licensed for use in Europe, Australia and the United States and a Yellow fever (YF-JE) chimeric vaccine candidate is nearing license in developed as well as developing countries[220]. The major outbreak of JE in Eastern UP during 2005, GoI introduced JE vaccination with SA-14-14-2 vaccine in phased manner starting form 2006. 132 districts have already been brought under JE vaccination as part of Universal Immunization Programme (UIP). Out of 62 new districts proposed to be covered during 2012-13 and 2013-14, 16 districts have already been covered under JE vaccination during 2012-13[62].

There is no vaccine to prevent human infection by Dengue virus. Personal protection and the environment management of mosquitoes are important in preventing illness. Prevent access of mosquitoes to an infection person with a fever. Protect ourselves from mosquitoes bites at all times in Dengue areas. Use mosquito repellent Permethrin can be applied to clothing, shoes, camping gear and bed netting. Use 10% repellent to apply our skin. The mosquito that carry the Dengue

virus typically live in and around houses, breeding in standing water that can collect in such things as used automobile tires. Reduce the breeding habitat to lower mosquito populations.

There is no vaccine to prevent human infection by West Nile virus. Apply repellent containing permethrin, picaridin, oil of lemon eucalyptus or DEET when mosquito are active to exposed skin, the more active ingredients a repellent contain the longer ;it can protect from mosquito bites. Repellents may irritate the eyes and mouth, so avoid applying repellent to the hands of children. Consider staying indoors at dawn, dusk and in the early evening, which are peak mosquito biting times.

3.12 CONTROL MEASURES

Vector control measures have their limitations given the exophilic as well as exophagic tendencies of the proven vectors belonging to *Cx.Vishnui* group. Control measures should be implemented immediately. Vector control measures especially fogging the Malathion technical should be carried out immediately in the affected village, use of bed net, full sleeve clothes during evening hours should be promoted to prevent mosquito bites.

Outdoor resting habits and crepuscular nature, the vector control using indoor residual spray is technically not feasible. In addition due to the vast and enormous breeding habitats like perennial ponds, paddy fields and other water bodies larval control using ULV fogging (ultra low volume) is the only recommended method of vector control.

Many flaviviruses are human pathogens of global importance, but no clinically approved antiviral therapy is currently available to manage these diseases. Both pharmaceutical industry and academia have invested considerable efforts over the past decade on finding the flavivirus antiviral using modern drug discovery. Various high-throughput compatible target-based and cell-based assays have been developed and implemented.

SCOPE AND PLAN

4.1 SEROPREVALENCE STUDY

Dengue, Japanese Encephalitis and West Nile encephalitis, are the common viral diseases associated with high morbidity and mortality. They are the leading cause of endemic/epidemic viral encephalitis in Asia, including India, Thailand, Vietnam, Singapore, the Philippines, Taiwan, China, Korea, and Japan[221]. It is also one of several mosquito-borne flaviviruses, in addition to four serotypes of Dengue virus (DENV-1 to -4), that have experienced emergence and reemergence throughout the world, especially in the tropical regions[222,223]. Sequential infection by multiple cocirculating flaviviruses in the affected population confounds serodiagnosis[201] disease burden estimation[224] and the impact on pathogenesis[225].

Flavivirus infections elicit protective antibody responses primarily against the envelope (E) glycoprotein[201]. The E protein contains three structural and functional domains. E domain I (EDI) is an eight-stranded β-barrel; it contains two large insertion loops forming the elongate dimerization EDII and containing the highly conserved internal fusion peptide. EDIII has an immunoglobulin (Ig)-like structure and contains the primary receptor-binding motifs[226,227]. Murine monoclonal antibody (MAb) studies have demonstrated that EDI contains predominately type-specific nonneutralizing (non-Nt) epitopes, EDII contains cross-reactive epitopes eliciting both Nt and non-Nt antibodies, and EDIII contains the majority of the type-specific Nt epitopes[228,229-234].

There is not much data available on the seroprevalence of the flavivirus antibodies in the population of Tamil Nadu. A seroprevalence study will give us a clear picture about the exposure of the population to flavivirus. In the present study the serum and CSF samples collected from suspected cases of AES from Jan 2011 to December 2014 and based on the date of onset of illness the samples were tested for IgM antibody capture ELISA. The samples were collected at different age intervals 0-12 yrs, 13-19 yrs, 20-35 yrs, 36-50 yrs and above 50 yrs. The seroprevalence study

give us a clear picture about the age wise, gender wise, month wise, season wise and district wise distribution of flavivirus causing AES.

4.2 SCREENING OF AES PATIENTS FOR FLAVIVIRUS ETIOLOGY BY RT-PCR

The clinical diagnosis of flavivirus infections is not unambiguous due to unspecific symptoms varying from mild, febrile illness to viral hemorrhagic fever. Many of these flaviviruses have a common transmission vector and can co-circulate in the same area. All these factors make a precise identification of the pathogen difficult.

Today most diagnostic laboratories use serological assays for flavivirus testing. These tests are commonly based on the enzyme-linked immunosorbent assay (ELISA) and detect virus-specific IgM and IgG antibodies. Antibodies are undetectable prior to 5-7 days after onset of infection which hampers the usefulness of the serological methods. Molecular techniques, in contrast, can be used already in the acute phase and are known to be rapid, highly specific and sensitive.

Since the early 1990s, several group-specific and generic molecular assays for detection of flaviviruses have been developed as reviewed previously[235-237]. A number of attempts to detect several flaviviruses in a single reverse transcription-polymerase chain reaction (RT-PCR) assay have been made[238]. These assays vary in the selected target region, assay format and detection method. The highly conserved NS3 and NS5 genes have been used predominantly for flavivirus testing, mainly as nested, hemi-nested RT-PCR or SYBR green real-time PCR, without using a probe for sequence verification.

The scope of this study was to standardize a rapid, sensitive and reliable TaqMan probe-based quantitative RT-PCR (qRT-PCR) assay for simultaneous detection of several flaviviruses, using the conserved NS5 gene region. The introduction of Locked-Nucleic Acid (LNA) bases in the probe increases robustness, specificity and sensitivity of the assay and has been shown to allow quantification of the viral load.

In recent years, numerous molecular techniques utilizing a generic approach have been described for flavivirus diagnosis[239-254]. For identification of flaviviruses, molecular amplification methods targeting regions of highly conserved *NS3* and *NS5*genes have been widely described, predominantly in the format of nested or heminested RT-PCR. In general, these techniques present lower sensitivity than specific flavivirus approaches when evaluated during EQA exercises for the molecular diagnosis of different flaviviruses. However, in some cases[255] their performance is comparable[256]. The use of generic methods for the molecular diagnosis of flaviviruses is only advisable when post amplification identification techniques (e.g., sequencing and melting temperature) are performed afterwards.

Development of pan-flavivirus molecular diagnostic assays has intrinsic limitations owing to the genetic diversity among viruses of the genus. Although several attempts have been made to achieve universal detection of flaviviruses, the development of sensitive and specific pan-flavivirus assays remains an important task when addressing global health challenges.

Recently, some methods were developed for the detection and differentiation of two flaviviruses or a combination of flaviviruses and other arboviruses (i.e., Alphaviruses) sharing the same geographical distribution. These methods could be of interest to laboratories in endemic areas, but, again, a discrimination step must follow to reach an accurate diagnosis[257-261].

In the present study the CSF samples which were collected at an acute onset of illness subjected to pan-flavi conventional RT-PCR. The CSF samples were transported to laboratory and stored at -20. The samples were initially processed for the conversion of cDNA and the pan-flavi conventional RT-PCR was performed. Further the pan-flavi PCR positive samples were subjected to RT-PCR for JEV, DENV and WNV for the more sensitivity.

4.3 GENETIC CHARACTERIZATION, SEQUENCING AND PHYLOGENETIC ANALYSIS

The genus flavivirus of the family viridae comprises over 70 viruses, many of which, such as the Japanese Encephalitis (JE) virus, Dengue (DEN) viruses, and West Nile virus are important human pathogens[1,2]. Dengue and its severe and sometimes fatal forms, Dengue hemorrhagic fever and Dengue shock syndrome, alone affect nearly 80 million people a year[129,159]. As demonstrated in recent outbreaks of meningitis by West Nile (WN) virus in Algeria and Romania, viruses of this group sometimes cause serious public health concern in unexpected locations[262]. In the present study, the pan-flavi PCR positive samples were subjected to RT-PCR for JEV, DENV and WNV for the more sensitivity. The genetic characterization of the above flaviviruses was performed to find out the genotypic characterization and mutational events were analyzed during the study period.

The circulatory strains in Tamil Nadu will be come as Outcome of the study. The phylogenetic tree was constructed with the neighbor-joining method, with a bootstrap analysis of 1000 replicates, using MEGA software, version 2.1[263]. The Phylogram and slated cladogram will be constructed by pair-wise comparison of nucleotide sequences of the partial polypeptide genes which will be classified and all isolates into two different lineages.

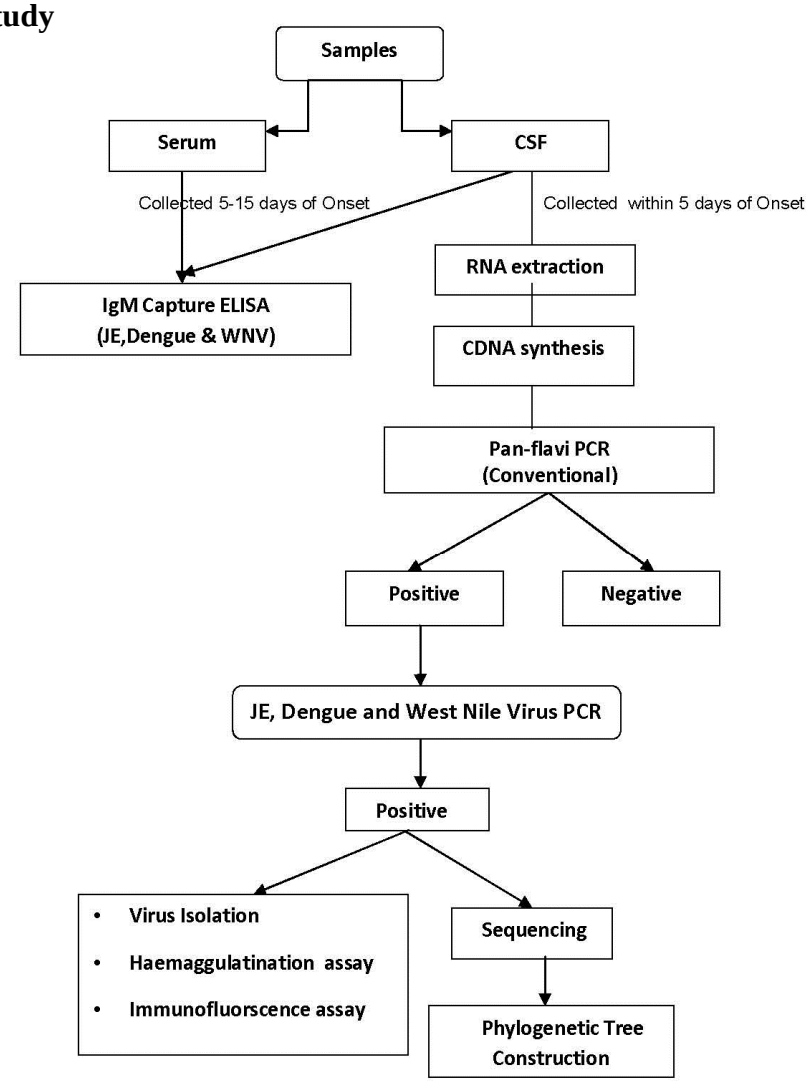
4.4 DEVELOPMENT AND STANDARDIZATION OF PAN-FLAVIVIRUS DETECTION SYSTEM (ELISA) USING PEPTIDE ANTIGEN TO ENABLE EARLY AND ECONOMICAL DETECTION OF FLAVIVIRUS INFECTION.

Several diagnostic systems are available for the detection of circulating flavivirus antibodies in patient serum[264,265]. However, no gold standard is available for immunological diagnosis. Since, PCR based diagnosis has become very common, it cannot be used in field conditions and requires trained manpower. A field based simple method to use pan-flavivirus detection system will be of great help specifically for low endemic areas to differentiate flavivirus infections from other viral infections[266].

In the present study, the conserved amino acid sequence among JEV, DEN and WNV were identified and it was custom synthesized. The peptide having conserved amino acid sequence was used to generate polyclonal antibodies and these antibodies were used to detect flaviviruses.

Similarly the fusion peptide was given to challenge in vivo animal model system. The Swiss albino mice were selected as an animal model system. The Swiss albino mice were injected with fusion peptide as per the gold standard of immunization protocol. The mice will be sacrificed in the end of the treatment and liver, heart, kidney and lungs were observed for the pathological findings induced by pan-flavi fusion peptide. The histological findings will be exposed the mutagenicity and antigenicity of pan-flvi conserved peptide.

Plan of the study



MATERIALS AND METHODS

The present study was carried out in the department of Virology, King Institute of Preventive Medicine & Research, Guindy, Chennai - 32 for the period from January-2011 to December-2014.

Study sample:

The cerebrospinal fluid (CSF) and serum samples received at King Institute of Preventive Medicine and Research (KIPM & R), Guindy, Chennai for the routine viral diagnosis of AES cases were obtained from various Government Hospitals as well as Private Hospitals in Tamil Nadu.

Inclusion Criteria:

Samples were collected from patients with Acute Encephalitis Syndrome (AES). AES is defined as a person of any age, at any time of year with the acute onset of fever and a change in mental status and/or new onset of seizures (sudden violent attack of an illness).

Exclusion Criteria:

The samples collected from the patient with other febrile illness.

Serum sample

The serum samples which are collected between 8 to 14 days from the date onset of illness were subjected to IgM capture ELISA. Blood samples collected from suspected cases, transported to lab in cold chain, serum separated and stored at -20°C till tested for ELISA.

Cerebrospinal fluid (CSF) sample

The CSF samples collected in acute stage of an infection (within 5 days from the date onset of illness) were included for the detection of flavivirus genome by

conventional RT-PCR and further processed for the detection of Japanese Encephalitis, Dengue and West Nile virus. So the conventional RT-PCR for the detection of these viruses was also standardized. The PCR positive samples were further used for the virus isolation, haemagglutination test and Immunofluorescence assay. The genetic characterization and phylogenetic analysis of PCR positive samples were also performed.

5.1 SEROPREVALENCE

The seroprevalence study was performed by detecting the presence of virus specific IgM antibody in serum and CSF samples collected from suspected AES cases.

JE IgM antibody capture ELISA in Serum & CSF

The JEV specific IgM antibody in serum and CSF was detected by NIV kit approved by CDC and the following materials were provided in the kit.

Materials

- Anti-human IgM Coated test strips – Micro wells are coated with anti-human IgM antibodies(μ chain specific) and post coated to block binding of non specific proteins as well to stabilize the coated antibody.(12x8 wells).
- JE Antigen-Ready to use.
- Biotinylated flavivirus cross reactive monoclonal antibody: (Hx-B)- Ready to use.
- Controls (Positive/Negative): Ready to use.
- Avidin-HRP: Ready to use as reagent. The solution to be protected from direct exposure to light.
- Wash Buffer Concentrate - 10x concentrate of phosphate buffered saline pH (7.2-7.6) with Tween 20 and preservative (0.1% Proclin). Crystallization may occur at low temperatures. To correct, incubate at 37°C until clear. Mix

well. Dilute one part wash buffer concentrate with 9 parts of distilled water. Diluted buffer may be stored for 1 week at 2-25^{o}C.

- Sample Dilution Buffer - Ready to use. Stable at 2-8^{o}C.
- Tetramethylbenzidine (TMB) Substrate: Ready to use. The solution to be protected from direct exposure to light.
- Stop Solution – Ready to use. 1 N- Sulphuric Acid. Stable at 2- 25°C.

Methodology

1. Dilute serum/plasma 1:100 or CSF 1:10 with sample dilution buffer.
2. Mix properly with vortex mixture.
3. Wash the coated/post coated wells thrice with wash buffer.
4. Transfer 50µl of diluted samples to the appropriate wells. Add 50µl reconstituted positive control/negative control to respective wells.
5. Keep the plates in a humidified box (A bread box with a soaked cotton/tissue paper) and incubate the plate at 37ºC for 1 hour.
6. At the end of incubation, wash the plate five times with wash buffer. Tap the plate after last wash on a tissue paper.
7. Add 50µl of JE antigen to each well.
8. Repeat step no 5 & 6.
9. Add 50µl of Hx-B to each well.
10. Repeat step no 5 & 6.
11. Add 50µl of Avidin-HRP to each well. Keep the plate in a humidified box (A bread box with a soaked cotton) and incubate the plate at 37ºC for 30 minutes.
12. Repeat step no 6.
13. Add 100µl of substrate (TMB/H_2O_2) to each well.

14. Incubate the plate in dark at room temperature. Wait for the development of colour, which normally develops in 10 ± 1 minutes.

15. Stop the reaction with 100µl of 1N sulphuric acid (H_2SO_4).

16. Measure the absorbance at 450nm within 10 minutes.

Quality control

Each kit is supplied with one Positive control and one Negative control. The controls are mainly used for validation of the test procedure and the interpretation of the results.

Expected values are given below

Positive: OD values ≥ 0.5.
Negative: OD value ≤ 0.18.

Interpretation of the results

If OD value of sample tested exceeds OD of Negative control by a factor 5.0 (Sample OD ≥ Negative OD x 5.0), the sample should be considered as "Positive" for JEV infection.

Dengue IgM antibody capture ELISA in Serum & CSF

The Dengue specific IgM antibody was detected by NIV kit and the following materials were provided in the kit.

Materials

- Anti-human IgM Coated test strips –Micro wells are coated with anti-human IgM antibodies (µ chain specific) and post coated to block binding of non specific proteins as well to stabilize the coated antibody.
- Dengue Antigen (Recombinant) – Ready to use.

- Biotinylated flavivirus cross reactive monoclonal antibody: (Hx-B) - Ready to use.
- Controls (Positive/Negative): Ready to use.
- Avidin-HRP: Ready to use as reagent. The solution to be protected from direct exposure to light.
- Wash Buffer Concentrate - 10x concentrate of phosphate buffered saline pH (7.2-7.6) with Tween 20 and preservative (0.1 % Proclin T). Crystallization may occur at low temperatures. To correct, incubate at 37°C until clear. Mix well. Dilute one part wash buffer concentrate with 9 parts of distilled water. Diluted buffer may be stored for 1 week at 2-25^{o}C.
- Sample Dilution Buffer - One bottles, 60 ml. Ready to use. Stable at 2-8^{o}C until expiry.
- Tetramethylbenzidine (TMB) Substrate : Twelve ml of TMB/H_2O_2 substrate is provided. Ready to use. The solution to be protected from direct exposure to light.
- Stop Solution – 1 bottle, 12ml ready to use. 1 N- Sulphuric Acid. Stable at 20 - 25℃ till expiry.

Methodology

Ensure all reagents are equilibrated to room temperature (20-25°C) before commencing assay.

1. Dilute serum/plasma 1:100 or CSF 1:10 with sample dilution buffer.
2. Mix properly with vortex mixture.
3. Wash the coated/post coated wells thrice with wash buffer.
4. Transfer 50µl of diluted samples to the appropriate wells. Add 50µl reconstituted positive control/negative control to respective wells.

5. Keep the plates in a humidified box (A bread box with a soaked cotton/tissue paper) and incubate the plate at 37ºC for 1 hour.
6. At the end of incubation, wash the plate five times with wash buffer. Tap the plate after last wash on a tissue paper.
7. Add 50µl of antigen to each well.
8. Repeat step no 5 & 6.
9. Add 50µl of Hx-B to each well.
10. Repeat step no 5 & 6.
11. Add 50µl of Avidin-HRP to each well. Keep the plate in a humidified box (A bread box with soaked cotton) and incubate the plate at 37 ºC for 30 minutes.
12. Repeat step no 6.
13. Add 100µl of substrate (TMB/H_2O_2) to each well.
14. Incubate the plate in dark at room temperature. Wait for the development of colour, which normaly develops in 10 ± 1 minutes.
15. Stop the reaction with 100µl of 1N sulphuric acid (H_2SO_4).
16. Measure the absorbance at 450nm within 10 minutes.

Quality control

Each kit is supplied with one Positive control and one Negative control. The controls are mainly used for validation of the test procedure and the interpretation of the results.

Expected values are given below

Positive: OD values $\geq$ 0.5
Negative: OD value $\leq$ 0.18

Interpretation of the results

If OD value of sample tested exceeds OD of Negative control by a factor 3.0 (Sample OD ≥ Negative OD x 3.0), the sample should be considered as "Positive".

West Nile IgM antibody capture ELISA in Serum and CSF

The West Nile specific IgM antibody was detected by Panbio kit (Inverness Medical Innovations Australia Pty Ltd, Australia) approved by CDC,Atlanta and the following materials were provided in the kit.

Materials

- Anti-human IgM Coated Microwells - (Assay Plate**)** Microwells are coated with polyclonal sheep anti-human IgM antibodies (12x8 wells). Ready for use.

- West Nile Virus Antigen (NY99 strain) - Ready for use. Inactivated WNV antigen in phosphate buffered saline (pH 7.0-7.4) with glycerol, preservatives (0.1% Proclin™, 0.05% gentamycin sulphate) and protein stabilizers. Stable at 2-8ºC until expiry.

- Wash Buffer Concentrate - 20x concentrate of phosphate buffered saline (pH 7.2-7.6) with Tween 20 and preservative (0.1% Proclin™). Crystallization may occur at low temperatures. To correct, incubate at 37ºC until clear. Mix well. Dilute one part wash buffer concentrate with 19 parts of distilled water. Diluted buffer may be stored for one week at room temperature (20-25ºC).

- Sample Diluent - Ready for use. Tris buffered saline (pH 7.2-7.6) with preservatives (0.1% Proclin™) and additives. Stable at 2-8ºC until expiry.

- HRP Conjugated Monoclonal Antibody Tracer - Ready for use. Horseradish peroxidase conjugated mouse monoclonal antibody (to flavivirus E-glycoprotein) tracer with preservative (0.1% Proclin™) and protein stabilizers. Stable at 2-8ºC until expiry.

- Tetramethylbenzidine TMB - Ready for use. A mixture of 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide in a citric-acid citrate buffer (pH 3.5-3.8). Stable at 2-8ºC until expiry.

- Positive Control - 400µL human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate). Stable at 2-8ºC until expiry.

- Cut-off Calibrator - 600µL human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate). Stable at 2-8ºC until expiry.

- Negative Control - 400µL human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate). Stable at 2-8ºC until expiry.

- Stop Solution - Ready to use. 1M Phosphoric acid. Stable at room temperature (20-25ºC) until expiry.

Methodology

Ensure all reagents are equilibrated to room temperature (20-25ºC) before commencing assay. Performing the assay outside the time and temperature ranges provided may produce invalid results. Assays not falling within the established time and temperature ranges must be repeated.

1. Dilute controls (negative, positive and calibrator) and the serum sample 1:100 with sample diluent and the CSF sample 1:10.
2. Calculate the number of wells accordingly mix equal volume of West Nile antigen and Mab tracer in a sterile vial. Mix it properly using vortex mixer and incubate at room temperature for 1 hour.
3. Within 10 minutes after mixing the MAb Tracer and antigen, pipette 100µL diluted sample and controls into the respective microwells of the assay plate.
4. Cover plate and incubate for 60 minutes at 37 ºC ± 1ºC.
5. Wash six times with diluted wash buffer.
6. Cover the plate and incubate for 1 hour at37°C ± 1°C.

7. Wash six times with diluted wash buffer.

8. Mix the antigen-MAb tracer solution before transfer. Pipette 100µl of antigen-MAb complexes to the appropriate wells of the assay plate.

9. Cover plate and incubate for 1 hour at 37°C ± 1°C.

10. Wash six times with diluted Wash Buffer.

11. Pipette 100µl TMB into each well.

12. Incubate for 10 minutes at room temperature (20 - 25ºC), timing from the first addition. A blue color will develop.

13. Pipette 100µl of stop solution into all wells in the same sequence and timing as the TMB addition. Mix well. The blue color will change to yellow.

14. Within 30 minutes read the absorbance of each well at a wavelength of 450nm with a reference filter of 600-650nm.

Calculation

(1) Calculate the average absorbance of the triplicates of the cut-off calibrator. This is the cut-off value.

(2) An index value can be calculated by dividing the sample absorbance by the cut-off value (calculated in step 1 above).

$$\text{Index Value} = \frac{\text{Sample Absorbance}}{\text{Cut-off Value}}$$

Alternatively

(3) Panbio Units can be calculated by multiplying the index value (calculated in step 2 above) by 10.

Interpretation of the results

If the panbio units of sample tested exceeds 11, the sample should be consider as positive for West Nile virus infection.

5.2 Pan-flavi PCR

The conventional PCR was performed in the CSF sample collected at acute onset of illness (sample collected within 5 days from the date of onset of illness). The samples were collected and stored at -20°C till the process. RNA extraction from CSF samples were done using Qiagen kit. cDNA was synthesized using random primers and PCR was performed first with flavivirus specific primer and the positives were then tested with primers specific for specific virus.

Materials for RNA extraction

The QIAamp Viral RNA kit (QIAGEN) was used for the extraction of RNA from CSF samples and the following materials were provided in the kit

- QIAamp mini spin columns
- Collection tubes (2mL)
- Viral lysis Buffer (AVL)
- Wash Buffer AW1 (concentrate)
- Wash Buffer AW2 (concentrate)
- Elution Buffer (AVE)
- Carrier RNA (poly A)
- 99% Ethanol
- 1.5mL micro centrifuge tubes
- Micro centrifuge

Miscellaneous – Pipette (1000µl, 100µl), disposable sterile tips, tissue papers

Methodology

a) Preparation of viral lysis buffer

- Add 310µl of AVE buffer to one tube of lyophilized carrier RNA (310µg) and dissolve thoroughly. Aliquot 28µl (for 5 reactions) and store at -20°C.
- Aliquot 560µl of AVL buffer and store at room temperature.
- Always prepare AVL buffer freshly, add 5.6µl of AVE+ Carrier RNA (kept at -20°C) to 560µl of AVL buffer (kept at room temperature).

b) Preparation of AW1 buffer

Buffer AW1 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol as indicated on the bottle. Store the buffer at room temperature.

c) Preparation of AW2 buffer

Buffer AW2 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol as indicated on the bottle. Store the buffer at room temperature.

Note: All centrifugations steps are at room temperature.

- Buffer AVL + carrier RNA stored at 2-8^{o}C forms a precipitate which must be re dissolved by warming at 80°C) for not more than 5 minutes. Cool to room temperature before use.
- Do not warm Buffer AVL + carrier RNA solution for more than 6 times, hence preferably aliquot in small quantities.

RNA extraction procedure:

- Pipette 560µl of prepared Buffer AVL containing carrier RNA into a 1.5ml micro centrifuge tube. (If the sample volume is larger than 140µl then increase the amount of buffer AVL or carrier RNA proportionally, e.g. 280µl sample will require 1120µl of buffer AVL or carrier RNA).
- Add 140µl of sample. Vortex - spin for 15 sec.
- Incubate at room temperature for 10 minutes
- Briefly centrifuge the 1.5ml micro centrifuge tube to remove drops from the inside of the lid.
- Add 560µl of ethanol (96-100%) to the sample and mix by pulse overtaxing for 15 sec. briefly centrifuge to remove drops from inside the lid.
- Carefully apply 630µl of solution from step 5 to column. Centrifuge at 8000 rpm for 1 min. Place column into a clean 2ml collection tube and discard the tube containing the filtrate.
- Repeat step 6.
- Add 500µl of Buffer AW1.Cetrifuge at 8000 rpm for 1 minute. Place column into a clean 2ml collection tube and discard the tube containing the filtrate.
- Add 500µl of Buffer AW2.Centrifuge at full speed (14000rpm) for 4 minutes.
- Place the column in a clean 2ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 minute.
- Place the column in a clean 1.5ml micro centrifuge tube. Add 60µl of Buffer AVE, incubate at room temperature for 1 minute. Centrifuge at 8000rpm for 1 minute.

In case of storage, store RNA at -20°C or -70°C till further use.

Synthesis of cDNA

The total viral RNA was reverse transcribed to complementary DNA using reverse transcriptase core kit developed by Eurogentec, Belgium and the following materials were provided in the kit.

Materials

- 10x reaction buffer (black cap): one bottle of RT reaction buffer KCl and Tris-HCl
- EuroScript reverse transcriptase (white cap): One tube of Moloney Murine leukemia virus reverse transcriptase, 3750U at 50 U/µl.
- RNase Inhibitor (purple cap): One tube of RNase inhibitor 2400U/ µl.
- 2.5mM dent mix(green cap): One tube of dATP,dCTP,dGTP and dTTP in autoclaved,deionized water titrated with NaOH to pH 7.0.
- 25mM $MgCl_2$ (orange cap): One tube of 25mM $MgCl_2$
- Oligo d(T)15VN (yellow cap): One tube containing 50µM oligodeoxynucleotides of sequence $d(T)_{15}VN$ in 10nm Tris-HCl, pH 8.3.
- Random Nonomers (pink cap): One tube containing 50mM short oligonucleotides of random sequence ($d(N)_9$) in 10mM Tris-HCl, pH 8.3.
- RNase free water(plain cap): One tube of DEPC water.

Template

The RNA extracted from the CSF samples of suspected cases and stored at -20°C. The prototype virus (JEV, DENV and WNV) obtained from NIV raised in Vero cell line and the RNA was extracted from the cell culture fluids. It was used as a positive control for each run of the PCR.

Methodology

Thaw all required reagents necessary for the RT step completely and put them on ice, except for the EuroScript, which should be kept in the freezer until require for use. Mix all reagents well by inversion and spin them down prior to pipetting.

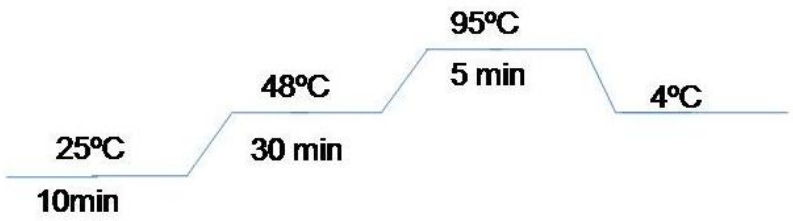
Preparation of RT reaction mix (10µl for 1x)

10x reaction buffer	-	2µl
25 mM $MgCl_2$	-	4µl
2.5mM dNTP	-	4µl
Random nonamer	-	1µl
RNAse Inhibitor	-	0.4µl
EuroScript RT	-	0.5µl
RNase free water	-	6.1µl
Template	-	2µl
Total mix	-	20µl

- Add all components together except for the template. Mix thoroughly by inversion. Spin down.
- Add the template to individual reactions, gently mix by inversion and spin down.
- A negative control containing no RNA template should always be included.
- Program the thermo cycler with the following cyclic conditions

Initial step	-	10 min 25°C
Reverse Transcriptase step	-	30 min 48°C
Inactivation of RT enzyme	-	5 min 95°C

Figure 5.1: Cyclic condition for cDNA synthesis



- The synthesized cDNA was stored at -20°C.

Materials for Pan-flavi PCR

- cDNA synthesized from CSF samples
- Positive control (Prototype virus raised in Vero cell line)
- Primers sequence given in Table 5.1
- Phusion High-Fidelity PCR Master Mix with HF Buffer
- Nuclease free water
- 0.2ml PCR tubes
- Pipettes (l-10µl, 20-200µl, 1000µl)
- gloves (powder free)
- 1.5ml microtubes (nuclease free)
- 0.6ml microtubes (nuclease free)
- Filter barrier tips (p10 to p1000)
- Thermal Cycler
- Gel casting tray
- Electrophoresis chamber with power supply
- UV Transilluminator
- 2% Agarose gel in 0.5% TBE
- Ethidium Bromide
- Gel loading buffer (50% glycerol, 25% Bromophenol blue and >25% Xylene Xyanole)
- Molecular weight marker (100-1000 DNA ladder)

Methodology for Pan-flavi PCR

One Step PCR Reaction Mix

25µl reaction volume containing 12.5µl of Phusion high-fidelity PCR master mix with HF buffer, 1µl of forward and reverse primers, 7.5µl of Nuclease free water and 3µl of cDNA was taken into the reaction mix.

Reaction Cycle

The thermal cycler was programmed with the cyclic condition illustrated in the figure 5.2.

Figure 5.2: Cyclic condition for pan-flavi PCR

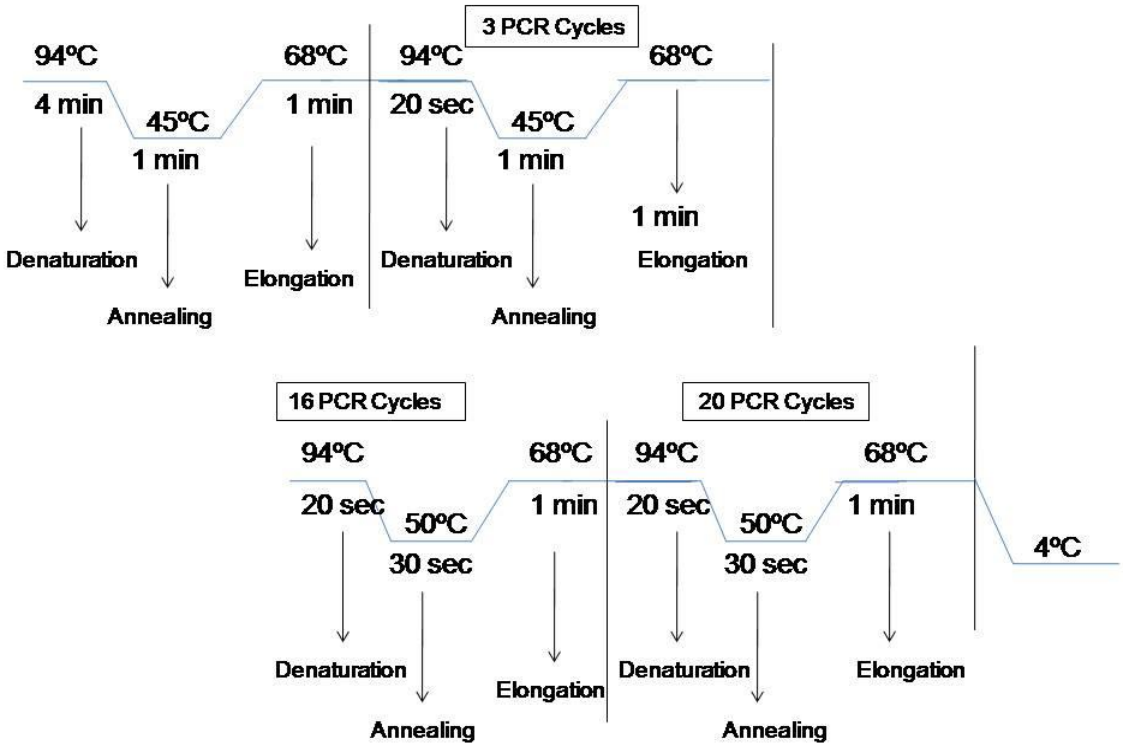


Table 5.1: Primer sequence for Pan-flavi, JE, Dengue and WNV PCR

Primer Name	Sequence (5'- 3')	Base pair
Pan-flavi PCR FU1(F)[250]	TACAACATGATGGGAAAGAGAGAGAA	655
cFD4(R)	ACCACACAATCATCTCCGCT	
JEP-1(F)[267]	CACAACGAGAAGCGAGCTGATAGTA	241
JEP-2(R)	CCCCAACTTGCGCTGAATAATTCCC	
Pan Dengue PCR[268] (MD1-D2)	TCAATATGCTGAAACGCGAGAGAAACCG	511
D2	TTGCACCAACAGTCAATGTCTTCAGGTTCC	
Dengue 1 (MD1/MTS1-)	CCCGTAACACTTTGATCGCT	208
Dengue 2 (MD1-TS2)	CGCCACAAGGGCCATGAACAG	119
Dengue 3(MD1-TS3)	TAACATCATCATGAGACAGAGC	288
Dengue 4 (MD1/MTS4-5)	TTCTCCCGTTCAGGATGTTC	260
West Nile (F)[269]	GCCGGGCTGTCAATATGCTAAAA	396
West Nile (R)	GCACTGGTCAAGGTCCCTAGTTC	

Methodology for JE PCR

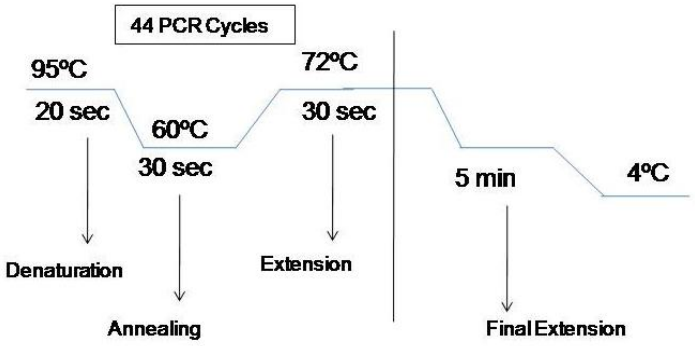
One Step PCR Reaction Mix

25µl reaction volume containing 12.5µl of Phusion high-fidelity PCR master mix with HF buffer, 1µl of forward and reverse primers, 7.5µl of Nuclease free water and 3µl of cDNA was taken into the reaction mix.

Reaction Cycle

The thermal cycler was programmed with the cyclic condition illustrated in the figure 5.3.

Figure 5.3: Cyclic condition for JE PCR



Methodology for Dengue PCR

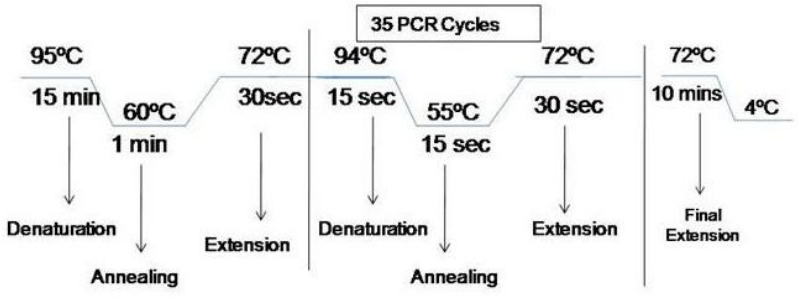
One Step PCR Reaction Mix

25µl reaction volume containing 12.5µl of Phusion high-fidelity PCR master mix with HF buffer, 1µl of forward and reverse primers, 7.5µl of Nuclease free water and 3µl of cDNA was taken into the reaction mix.

Reaction Cycle

The thermal cycler was programmed with the cyclic condition illustrated in the figure 5.4.

Figure 5.4: Cyclic condition for Dengue PCR



For Dengue genotyping

25µl reaction volume containing 12.5µl of Phusion high-fidelity PCR master mix with HF buffer, 1µl of forward primer(common for all genotype) and 1µl of reverse primer(specific for each genotype illustrated in Table 5.1) ,7.5µl of Nuclease free water and 3µl of cDNA was taken into the reaction mix. The cyclic condition was same as shown in figure 5.4.

Methodology for West Nile PCR

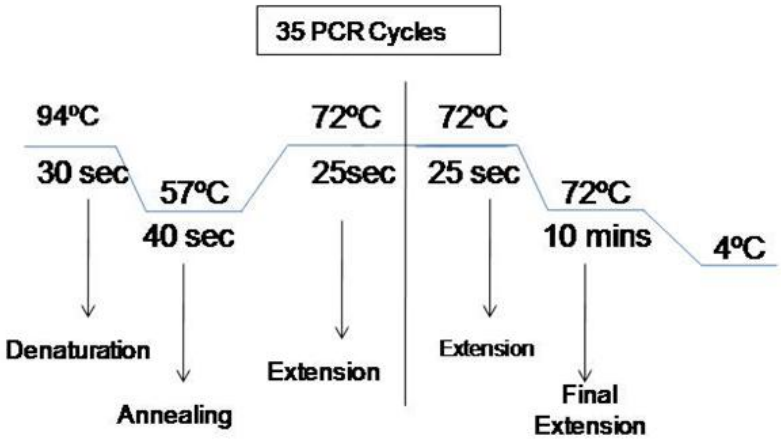
One Step PCR Reaction Mix

25µl reaction volume containing 12.5µl of Phusion high-fidelity PCR master mix with HF buffer, 1µl of forward and reverse primers, 7.5µl of Nuclease free water and 3µl of cDNA was taken into the reaction mix.

Reaction Cycle

The thermal cycler was programmed with the cyclic condition illustrated in the figure 5.5.

Figure 5.5: Cyclic condition for WNV PCR



Product Analysis by Agarose Gel Electrophoresis

- 2g of Agarose was dissolved in 100ml 0.5XTBE buffer and melted using hotplate. Once completely melted, 2µl of Ethidium bromide was added and mixed completely.
- The melted agarose was poured on to the gel plate with comb and allowed for solidification for at least 1 hour.
- After solidification the gel plate was kept in electrophoresis tank containing 0.5X TBE buffer and comb was removed.
- 8µl of PCR product was mixed with 2µl of gel loading dye and loaded into gel. The electrophoresis apparatus was run for 40 minutes.
- Once electrophoresis completed, the gel was visualized under UV transilluminator and the image captured using gel documentation system.

5.3 Genetic characterization of the positive samples

RT-PCR using flavivirus primer was performed and the PCR product was sequenced according to the standard protocols. Amplicons were purified using QIAquick PCR purification kit (Qaigen) and subjected to cycle sequencing using ABI Prism Big Dye terminator V3.1 cycle sequencing kit. Post-cycle sequencing purification was done using DyeEx2.0 spin kit (Qiagen). Sequencing was performed on ABI Prism 310 and sequence alignment and phylogenetic analyses will be done using Mega version 6.

Virus Isolation

The virus isolation was performed for the PCR positive CSF samples in Vero cell line (African green monkey kidney). The cell line was obtained from National Centre for Cell Science (NCCS), Pune and maintained in tissue culture lab facility available in the department of virology, KIPM & R. The following materials were used for the preparation of media and tissue culture work.

Materials for media preparation and tissue culture work

Tissue Culture flasks 25cm^2

5ml, 10ml, pipettes

Minimum Essential Medium (MEM)

Trypsin Phosphate buffered saline Versene Glucose (TPVG)

Beaker with 1% hypo chloride solution

Gloves

Spirit

Cotton

Label pad, Marker

Preparation of ingredients for Media

Penicillin and Streptomycin: (Conc.100IU of penicillin and 100µg of streptomycin)

1 X 10^6 units of crystalline penicillin and 1g of streptomycin were dissolved in 100ml of PBS. 1ml of this stock was added to 100ml of medium to give a final concentration of 100IU penicillin and 100µg of streptomycin. Stored at -20^oC.

Kanamycin Acid Sulphate (Conc:20µg/ml)

1g of kanamycin was dissolved in 50ml millipore double distilled water and mixed well to a final concentration of 20µg/ml and stored at -20^oC

Fungizone : Amphotericin B – 50mg (Conc:20µg/ml)

50mg of amphotericin B was dissolved in 5ml of sterile Millipore distilled water and 1ml of this stock was diluted to 100ml of sterile Millipore distilled water to a final concentration of 20µg/ml and stored at -20°C.

3% L-Glutamine

6g of L-Glutamine was dissolved 200ml of sterile millipore distilled water and mix well. It was filtered through 0.22 micron membrane and distributed as 5ml in polypropylene vial and stored at -20°C.

7.5% Sodium-bi-carbonate solution

22µg of Sodium Bicarbonate was dissolved in 200ml of sterile millipore distilled water and filtered through Whatmann filter paper No.4 and autoclaved. It was stored at +4°C.

Fetal Bovine Serum

Fetal bovine serum was thawed at room temperature and inactivated at 56°C in water bath for ½ hour and cooled at room temperature. If floating particles were observed it was filter through Seitz filter and stored at -20°C.

Trypsin, PBS, Versene, Glucose solution : (TPVG)

Phosphate Buffered Saline

NaCl A.R	-	8g
KCl A.R	-	0.2g
Na_2HPO_4 A.R	-	2.88g
KH_2PO_4 A.R	-	0.2g
pH	-	7.4

All the above ingredients were dissolved in 1000ml of sterile distilled water and filtered through whatmann filter paper No.4 and autoclaved.

2% Trypsin

2g of trypsin was dissolved in 100ml sterile millipore distilled water stirred for 30 minutes with help of magnetic stirrer. The solution was filtered through membrane filter and stored at -20°C.

0.2% EDTA (Versene)

200mg of EDTA was dissolved in 100ml of sterile millipore distilled water and autoclaved.

10% Glucose A.R.-10ml

1g of glucose was dissolved in 10ml of sterile millipore distilled water and filter through whatmann filter paper and autoclaved.

TPVG	-	1000ml
PBS	-	840ml
2% Trypsin	-	50ml
0.2% EDTA	-	100ml
10% Glucose	-	5ml
P&S	-	5ml

All the ingredients were mixed and pH was adjusted to 7.4 and stored at -20°C.

0.4% Phenol Red

0.04g of Phenol red was dissolved in 10ml of double distilled water and autoclaved.

Trypan blue for cell counting (0.1%)

0.1g of Trypan blue was dissolved in 100ml of phosphate buffered saline, filtered through whatmann filter paper No.4 and store at 4^{o}C.

Methods for the media preparation

The following table illustrates the composition of media and its preparation.

Table 5.2: MEM Preparation Procedure

Ingredients	10% Growth Media	2% Maintenance Media
MEM	861ml	941ml
P&S (Penicillin & Streptomycin)	1ml	1ml
Phenol red (0.4%)	1ml	1ml
Kanamycin	1ml	1ml
Fungizone	1ml	1ml
3% L-Glutamine	10ml	10ml
Fetal Bovine Serum	100ml	20ml
2.2g Sodium bicarbonate	20ml	20ml
Hepes buffer (IM)	5ml	5ml
Total volume	1000ml	1000ml

All the ingredients were mixed well by shaking gently and pH was checked and adjusted to 7.2 to 7.4.

Maintenance of Cell Lines

- Tissue culture bottles that showed confluent monolayer were selected by observing them under an inverted microscope.
- Growth medium was removed from the bottle, washed with PBS/MEM without FCS and 5ml of TPVG (for 25cm^2) was added dispersing evenly on the monolayer and left in contact with the cells for 2-3 minutes.
- TPVG was removed and the bottle was incubated at 37°C, until all the cells were detached from the surface.
- The cells were re-suspended in 5ml of growth media. The suspension was aspirated few times to break cell clumps.
- The cell concentration was determined by counting the cells in haemocytometer.

Cell Counting

- A 0.2ml of the cell suspension was diluted in 0.2ml of trypan blue (0.1% trypan blue).
- It was mixed well with pipette and sufficient volume was aspirated to fill haemocytometer immediately.
- The viable cells (nonviable cells are stained blue and viable cells remain unstained) were counted in each of the four corners of both chambers, omitting cells lying on the top and to the left.
- If cell clumping was observed, it was discarded and original cell suspension was re-suspended.
- The total number of cells in the suspension was calculated using the following formula.

Average cell count x dilution factor x 10^4 = - lakhs/ml

Or $C_1 = t \times tb \times ¼ \times 10^4$

where	C_1	=	Initial cell concentration per ml
	t	=	total viable cell count of 4 squares
	tb	=	Correction to the trypan blue dilution
	1/4	=	correction to give mean cells per corner square.
	10^4	=	conversion factor for counting chamber.

- Based on the cell count, sterile flasks/tubes were seeded and incubated at 37°C.
- Cell lines were not passaged beyond 15 times.

For preparation of 25cm^2 flask

Growth medium was dispensed to fresh sterile cell culture flasks to be seeded with cells (9ml/25cm^2 flask). The cell suspension was seeded to the TC flasks at 1.0 x 10^5 cells/flask. Each flask was labeled with cell name, passage number and date of passage and incubated in 5% CO_2 environment for 48 hours.

Virus inoculation

- 48 hours old healthy, confluent, vero cell line grown in 25 cm^2 tissue Culture flasks were choosen.
- Media was removed from flask with sterile pipette.
- Add 50µl of CSF sample to 25cm^2 flask using sterile pipettes. Inoculum was allowed to adsorb for 30 minutes at 37C.
- Add 10ml of maintenance media contain 2% fetal bovine serum to each flask and incubate at 37°C.
- Flasks were observed daily for cytopathic effect (CPE).

Haemagglutination test (HA)

The JE, Dengue and West Nile viruses are able to agglutinate the RBC of goose and it is pH dependent. So it is necessary to titrate the antigen in different pH solution. The optimum pH for haemagglutination was determined.

Materials

- Virus adjusting diluents (VAD) of varying pH
- Borate saline (pH 9)
- 0.4% Bovine albumin in borate saline (BABS) pH 9
- Goose erythrocytes in Alsever's solution
- 0.9% Normal saline
- Acid citrate dextrose
- West Nile Virus antigens
- 96 well 'U'-bottom plate

- Miscellaneous- Cooling centrifuge, centrifuge tubes, pipettes (50µl and 100µl), tips, polypropylene vials, test samples, tissue papers

Preparation of Reagents

Virus Adjusting Diluent (VAD)

Preparation of stock solutions

(A) 1.5M Sodium chloride (Nacl): 87.65g of Sodium chloride in 100ml of distilled water and made to 1L

(B) 0.5 M Boric acid: 30.92g of boric acid dissolved in 700ml hot distilled water and allowed to cool at room temperature, and made to 1L with double distilled water.

(C) Concentrated Sodium Hydroxide (NaOH): 500ml of double distilled water added to 500g of sodium hydroxide pellets and the final concentration is 18M

(D) 0.5M Dibasic sodium phosphate: 70.99g of dibasic sodium phosphate dissolved in 100ml of distilled water and made to 1L.

(E) 1M Monobasic sodium phosphate (NaH_2PO_4): 138.01g of monobasic sodium phosphate dissolved in distilled water and made to 1L.

Table 5.3: Preparation the stock solution to be added for VAD

STOCK SOLUTION	pH 6.0	pH 6.2	pH 6.4
1.5M sodium chloride	100ml	100ml	100ml
0.5M dibasic sodium hydrogen phosphate	32ml	64ml	112ml
1M monobasic sodium dihydrogen phosphate	184ml	169ml	144ml
Distilled water	1L	1L	1L

Borate Saline (pH **9.0)**

1.5M sodium chloride	-	160ml
0.5M boric acid	-	200ml
1.0M sodium hydroxide	-	47ml

The above mixture is made up to 2L with distilled water and pH adjusted to 9.0 with 1M sodium hydroxide or with 0.5M boric acid and stored in refrigerator.

0.4% Bovine Albumin in Borate Saline (BABS)

0.4g of bovine albumin dissolved in 1000ml of borate saline (pH 9.0) and kept in refrigerator for overnight. pH is adjusted to 9.0 using 1M sodium hydroxide.

Acid Citrate Dextrose

Sodium citrate	-	11.26g
Citric acid	-	40g
Dextrose	-	11.0g
Distilled water	-	500ml

Prepared solution was sterilized by autoclaving.

Normal Saline

9.0g of sodium chloride dissolved in 1000ml of distilled water and sterilized by autoclaving.

Alsever's Solution

Sodium citrate	-	4g
Sodium chloride	-	2.1g
Dextrose	-	10.25g
Citric acid	-	0.55g

Dissolved and made to 500ml with distilled water and steam sterilized

Goose Blood Collection

- 45ml of acid citrate dextrose taken in a syringe fitted with a 20 gauge needle.
- About 2.3ml of venous blood collected from the wing or the jugular vein.
- The blood transferred to flask containing 3-4ml of alsever's solution.
- The suspension was mixed and stored at 4°C.

Washing of Goose Erythrocytes

- In centrifuge tube required quantity of erythrocyte suspension was taken and centrifuged at 2000rpm for 6 minutes.
- The supernatant fluid and Buffy layer of WBC removed, without disturbing the packed RBC.
- 2ml of normal saline was added and mixed. Then the suspension centrifuged at 2000rpm for 6 minutes.
- Repeat the above steps.
- 10% suspension was made by removing the supernatant fluid and by adding sufficient quantity of saline based upon the volume of packed RBC.

Treatment of Sera

Test serum treated with acetone extraction procedure to remove the non-specific inhibitors, and adsorbed with goose erythrocytes to remove natural agglutinins.

Acetone Extraction of Sera

- 0.9ml of normal saline and 0.1ml of serum added to 15ml centrifuge tube.
- The tube placed in ice water bath and chilled acetone added and incubated for 5 minutes by mixing occasionally for precipitation.

- The tube centrifuged at 3000rpm for 6 minutes at 4°C, the supernatant was decanted and 10ml of fresh acetone was added and incubated for 60 minutes at 4°C.
- The suspension was centrifuged at 4°C for 6 minutes at 3000rpm. The supernatant was discarded and the tube dehydrated in vacuum desiccator for 1-2 hours.
- 1ml of borate saline was added in to tubes and mixed well for complete rehydration of suspension.
- The preparation was kept overnight at 4°C.

Adsorption of Sera with Goose Erythrocytes

- Acetone-extracted serum was kept in an ice bath and 0.1ml of washed goose erythrocytes was added to serum.
- The suspension was centrifuged at 3000rpm for 15 minutes at 4°C.
- The supernatant was separated in a 2ml polypropylene vial and stored in refrigerator until tested or stored at -20°C deep freezer for further use.

Protocol

- The 96 well 'U'- bottom plate labeled as shown in the figure 5.6.
- 50µl BABS (pH 9.0) added to each well except the neat column and plate chilled in fridge for few minutes.
- 100µl virus isolates were added to the neat column.
- 50µl from neat taken and two fold serial dilutions were made until the last well of that row and the last 50µl discarded.
- 2.4ml of VAD of pH ranging from 6.0, 6.2 and 6.4 were taken.

- 0.1ml of 10% RBC added to each tube and mixed well, hence that is 0.45% of RBC.
- BABS plus the cells suspended in respective VAD serves as cell control.
- The plate was shaken gently and incubated room temperature till the cells in the control wells settled down (about 30 minutes).
- The HA titre was recorded.

Figure 5.6: 96 well 'U'-bottom plate

	N	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
6.0												
6.2												
6.4												
C												

pH 6.0 pH 6.2 pH 6.4

N- Neat C- Control

Immunofluorescence Assay

The immunofluorescence assay was performed for the detection of virus from infected tissue culture fluids and the following materials were used for IFA.

Materials

. Immunofluorescent microscope
. Teflon coated slides
. Positive control
. Negative control
. Vero cell line

. 0.01M PBS

. Buffered glycerol

. Acetone

. Anti-Rabbit FITC conjugate (DAKO)

Reagents

Phosphate Buffered Saline (PBS)

Sodium Chloride	-	7.0g
Pottasium Chloride	-	0.2g
Pottasium Di Hydrogen Phosphate	-	0.2g
Di Sodium Hydrogen Phosphate	-	1.5g
pH	-	7.2

All the above ingredients were dissolved in 1000ml of distilled water and autoclaved.

Buffered glycerol

Glycerol	-	9ml
PBS	-	1ml
pH	-	7.2

Methodology

- The PCR positive CSF samples were inoculated in Vero cell line and incubated at 37°C.
- After 7 days CPE was observed the cells were scrapped and a smear was made on the marked area of glass slide.
- The slides were fixed with chilled acetone for 15 minutes at room temperature.

- 20µl of 1:10 flavivirus cross reactive monoclonal antibodies (NIV, Pune) were added on the smear and incubated in humidified box at 37°C for 30 minutes followed by several washes with PBS(pH 7.2).

- 20µl of 1:10 anti-mouse IgG FITC conjugate was added to the smear and incubated at 37°C in humidified box and incubated at 37°C for 30 minutes.

- Smear air-dried and mounted with buffered glycerol mounting medium and observed under immunofluorescent microscope for intracytoplasmic apple green fluorescence.

5.4 DEVELOPMENT AND STANDARDIZATION OF PAN-FLAVIVIRUS DETECTION SYSTEM

In the present study the conserved amino acid sequences among Japanese Encephalitis, Dengue and West Nile viruses were identified and the peptide having the conserved amino acid sequence was used to generate polyclonal antibodies in mice module and these antibodies were used to detect those flaviviruses.

Identification of flavi-specific epitope

Amino acid sequences of envelope protein of several flaviviruses were downloaded from NCBI databank. Amino acid sequences were edited and analyzed by the Laser gene software package V5.0 (DNASTAR Inc, USA). Multiple sequence alignments were carried out employing CLUSTAL W version 1.83.

Synthesis of peptide

The flavi-specific conserved amino acid sequence was found to be “DRGWGNGCGLFGKG”. The identified peptide is custom synthesized by Sigma-Aldrich Company. The purity and homogeneity of the synthesized peptide was analyzed by RP-HPLC, which can separate peptides of even one amino acid difference based on differences in surface hydrophobicity. The synthesized peptide was dissolved in triple distilled sterile water so as to achieve a final concentration of 1 mg/ml and stored at -20°C till used.

Structural and physico-chemical characterization of the peptide

The synthesized peptide was analyzed by reverse phase HPLC. MALDI-MS analysis of peptide will be done following the protocol of Brown and Lennon.

Predictive analysis of the peptide

Predictive structural analysis of the peptide was carried out using RasWin Molecular Graphics. Antigenicity, hydrophilicity and secondary structure of the peptide will be predicted using Protean^{TM}.

Animals and immunization

Mice used in this study were obtained from the animal facility of the KIPM, Chennai. Mice were housed, fed and used in experiments according to the guidelines set forth in the guidelines for the production of antibodies in laboratory animals (Animal Care and Use Program, University of California, Berkeley). Swiss Albino mice of 3-4 weeks old were immunized by the subcutaneous route with 50µg/ml concentration of peptide emulsified in with Freund's complete adjuvant. The mice (25-30g) were procured from the King Institute of Preventive Medicine and Research, Guindy, Chennai, India. The animals were housed separately in polypropylene cage at temperature of 23 ± 2^{o} C and 50-60% relative humidity, with a 12h light/dark cycle respectively, for one week before and during the commencement of experiment in the department of virology, KIPM & R, Guindy, Chennai. Animals were allowed to access standard rodent pellet diet and drinking water. Food was withdrawn 18-24h before the experiment, though water was allowed adlibitum and allocated to different experimental groups. All experimental procedures involving animals were conducted in accordance with the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). The study protocols were approved by Institutional Animal Ethics Committee (IAEC) of KIPM & R, Guindy, Chennai.

The animals were divided into 3 groups, each group constituting 5 mice.

Group-1: Control

Group-2: Immune peptide 100 mg/kg

Group-3: Immune peptide 50 mg/kg

The peptide was administered through subcutaneous injection. The mice were observed for acute toxicity up to 8 days.

The animals were boosted on days 28, 53 and 150 days. Sera were collected at 14 days after immunization.

Histopathological Studies

After the duration of the experiment, the tissues like heart, lungs, liver and kidney were fixed in 10% neutral formalin for 24 hours. Cut sections by razor. Wash the fixatives first with tap water and then in the distilled water. Dehydrate in graded alcohols. Clear in xylene. The tissues were embedded in paraffin wax, made block and cut sections of 5 micron thickness, stained and mounted the slides in D.P.X. Sections were dewaxed in xylene for 5 minutes. Hydrated to water through descending grades of alcohol series with two minutes intervals in each series. The sections were stained with in haematoxylin for 20-30 minutes. Place the slides in tap water for 5 minutes. Differentiated in acid- alcohol with a quick dip of slides into the solution. Again dip in tap water until the sections were counter stained with eosin for 3-5 minutes. The sections were dehydrated in ascending grades of alcohol series with 5 minutes intervals in each series, cleared completely in xylene for 1 minute mounted in DPX. Microphotographs were taken using a Topcon RE 200 camera (Japan) attached to the microscope (Olympus).

Standardization of Pan-flavi ELISA

Mice hyper-immune sera were used for recognition of peptide by ELISA. Briefly, wells of a microtitre plate were coated with 1µg of peptide and blocked with 5% skimmed milk powder solution in phosphate-buffered saline (blocking buffer). Antigen-coated wells were incubated for 90 minutes at 37°C with 100µl of mice serum diluted in blocking buffer. Serial two fold dilutions of sera starting with 1:10

were used. After washing with PBS containing 0.05% Tween 20, 100µl of horseradish peroxidase-labeled anti-mouse IgG antibody conjugate diluted 1:2000 was added to each well and incubated for 60 minutes at 37°C. The color reaction was developed with tetramethyl bezidine substrate.The reaction was stopped by addition of 1N sulfuric acid and OD was recorded at 490nm in a microplate ELISA reader. Pre-immune sera as well as sera raised against adjuvant alone were used with similar dilutions as controls.

Dot-ELISA

Mice hyper-immune sera were also used for detection of flaviviruses by dot-ELISA. Briefly, Nitrocellulose strips (NC strips) were coated with heat inactivated virus suspension of flavivirus epitope. The coated strips were blocked and kept standing in wells of micro titre plates filled with various dilutions of mice serum. The conjugate was added and results were analyzed. Pre-immune sera as well as sera raised against adjuvant alone were used with similar dilutions as controls. NC strips were dried and observed for the appearance of brown color dots.

Reagents used in the study

Analytical, tissue culture and molecular grade fine chemicals used in this study were obtained from following firms-Sigma chemical company, GIBCO BRL, Life technologies, USA, SRL India and Hi Media.

Viral RNA extraction kit was procured from Qiagen. Enzymes and molecular markers used in the study were obtained from Bio Labs New England inc.

Equipments used in this study

Name	**Manufacturer**
1. Inverted microscope	- Nikon
2. Immunofluorescence microscope	- Nikon
3. ELISA reader & washer	- Thermo lab system
4. Centrifuge	- Eppendorf and Remi
5. CO_2 Incubator	- Binder

6. Vortex mixer	-	Remi
7. Micropippettes	-	Gilson / Finn pipette
8. Thermocycler	-	Applied Biosystems
9. Gel documentation system	-	Alpha imager

Statistical analysis

The Data was statistically analyzed by the software SPSS.20 IBM version. Specific type distribution was assessed by ANOVA and post hoc Dunnet analysis were performed in various variables such as sex, age, month and positivity were performed. Degrees of freedom were set as 0.05 level. Comparison with different viral species was analyzed. Further Linear regression were performed for year wise analysis of significance in samples. Ms-Excel was used for the graphical plotting of variables in different year and seasons using standard error mean.

RESULTS AND ANALYSIS

6.1 SEROPREVALENCE

The suspected AES cases (collected after 4 days of onset) referred from different hospitals of Tamil Nadu were screened for Japanese Encephalitis, Dengue and West Nile viruses by IgM Capture ELISA. The positivity of JEV, DENV and WNV were tabulated in the table 6.1 and 6.2.

Table 6.1: Results of Serum IgM ELISA

Year	Total No. of samples screened	JE Positive	Dengue Positive	WNV Positive
2011	218	12	5	3
2012	505	35	14	2
2013	936	45	21	4
2014	900	35	20	4

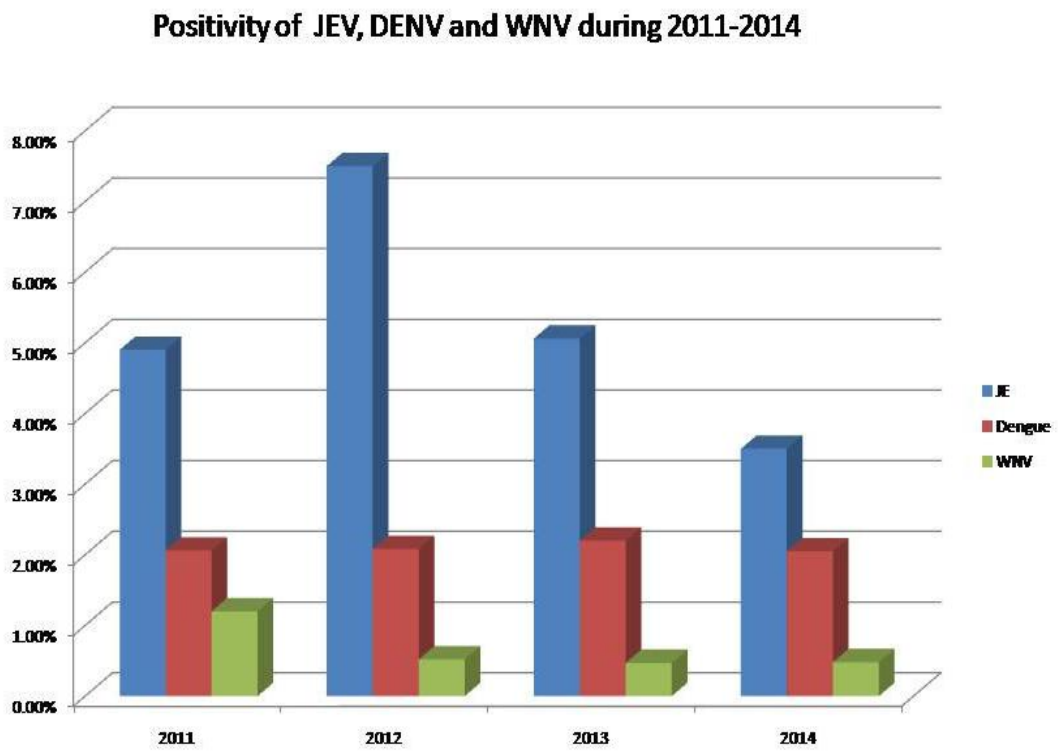
Table 6.2: Results of CSF IgM ELISA

Year	Total No. of samples screened	JE Positive	Dengue Positive	WNV Positive
2011	76	2	2	1
2012	294	12	7	2
2013	713	23	15	2
2014	709	10	17	2

In the study, the percentage positivity of JEV, DENV and WNV viruses were calculated and presented in the figure 6.1. Among the three viruses, JE virus showed higher prevalence than Dengue and West Nile viruses. Though the number of

samples increased during the study period there was not a significant difference in the percentage positivity shown in the figure 6.1.

Figure 6.1: Year wise Distribution of JE, Dengue and West Nile virus (2011-2014)



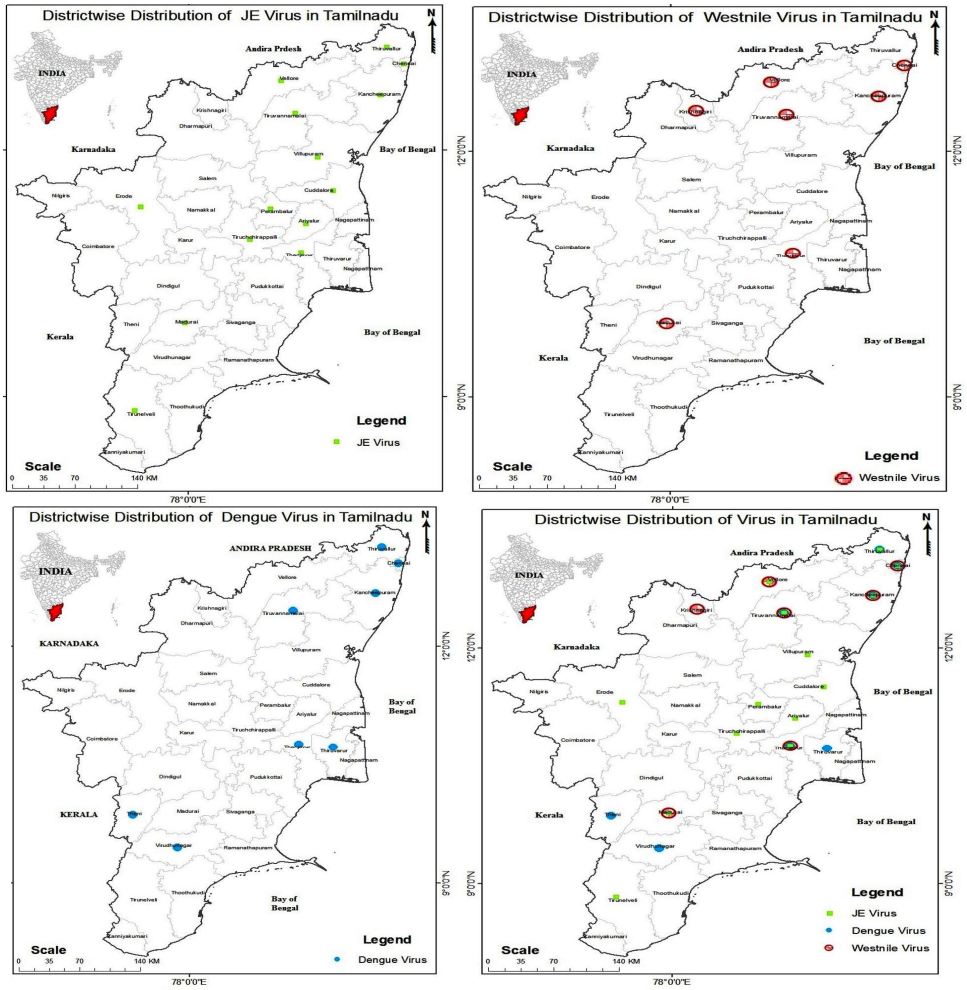
Highest percentage positivity was observed in the year 2012 and it was decreased afterwards. The similar trend was also observed for CSF samples regarding Dengue and West Nile viral infections.

Epidemiology and Incidence of flavivirus causing Acute Encephalitis Syndrome (AES) in Tamil Nadu

The suspected AES cases were screened for the incidence of flaviviruses such as Japanese Encephalitis, Dengue and West Nile viruses in various districts of Tamil Nadu during the period of 2011-2014. The district wise distributions of

positive cases were represented in the figure 6.1a. The positive cases of JE were investigated in fourteen districts of Tamil Nadu (Figure 6.1a).

Figure 6.1a: Tamil Nadu map showing District wise distribution of JE, West Nile and Dengue virus



Similarly the positive cases of Dengue virus were represented in seven districts and West Nile viruses were represented in eight districts of Tamil Nadu as shown in figures 6.1 and Table 6.3-6.5.

Table 6.3: District-wise distribution of Laboratory confirmed positive cases of JE from 2011-2014

S.No	District	2011	2012	2013	2014
1	Chennai.	2	9	13	7
2	Thiruvallur.	13	6	8	9
3	Kanchipuram.	5	7	10	6
4	Thiruvannamalai.	0	2	4	2
5	Villupuram.	0	1	0	2
6	Cuddalore.	0	1	2	0
7	Thanjavur.	1	2	4	1
8	Ariyalur.	0	1	1	0
9	Perambalur.	0	0	1	1
10	Vellore.	1	3	1	2
11	Trichy.	0	1	0	1
12	Madurai.	0	1	0	1
13	Thirunelveli.	0	0	1	2
14	Erode.	0	1	0	1

The maximum positive AES cases of JE and Dengue were reported in Chennai, Thiruvallur and Kanchipuram districts of Tamil Nadu. The higher positive AES cases of West Nile virus were explored in Thiruvannamalai, Chennai and Thiruvallur districts.

Table 6.4: Districtwise distribution of Laboratory confirmed positive cases of Dengue from 2011-2014

S.No	District	2011	2012	2013	2014
1	Chennai.	1	0	0	1
2	Thiruvannamalai.	1	0	1	1
3	Kanchipuram.	0	1	0	0
4	Vellore.	0	1	0	0
5	Krishnagiri	1	0	1	0
6	Madurai	0	0	1	1
7	Thanjavur	0	0	0	1
8	Cuddalore	0	0	1	0

Table 6.5: District-wise distribution of Laboratory confirmed positive cases of West Nile from 2011-2014

S.No	District	2011	2012	2013	2014
1	Chennai.	2	5	7	5
2	Thiruvallur.	2	3	3	2
3	Kanchipuram.	1	2	5	8
4	Thiruvannamalai.	0	2	2	1
5	Thanjavur.	0	1	2	0
6	Virudhunagar	0	0	1	3
7	Theni	0	1	1	1

6.3 AGE WISE DISTRIBUTION OF AES CASES

Samples representing different age groups such as 0-12, 12-18, 18-35, 35-55 and above 55 were segregated. The prevalence in different age groups were statistically analyzed and represented in the figure 6.2-6.4 by standard error mean. Among the various age groups, the positivity was predominantly seen in the pediatric age group during the study period. Few positive cases were observed in young adults. During the period 2013 and 2014, positivity was observed in adults and geriatric age group.

Figure 6.2: Epidemiological study of JEV during the year 2011-2014.

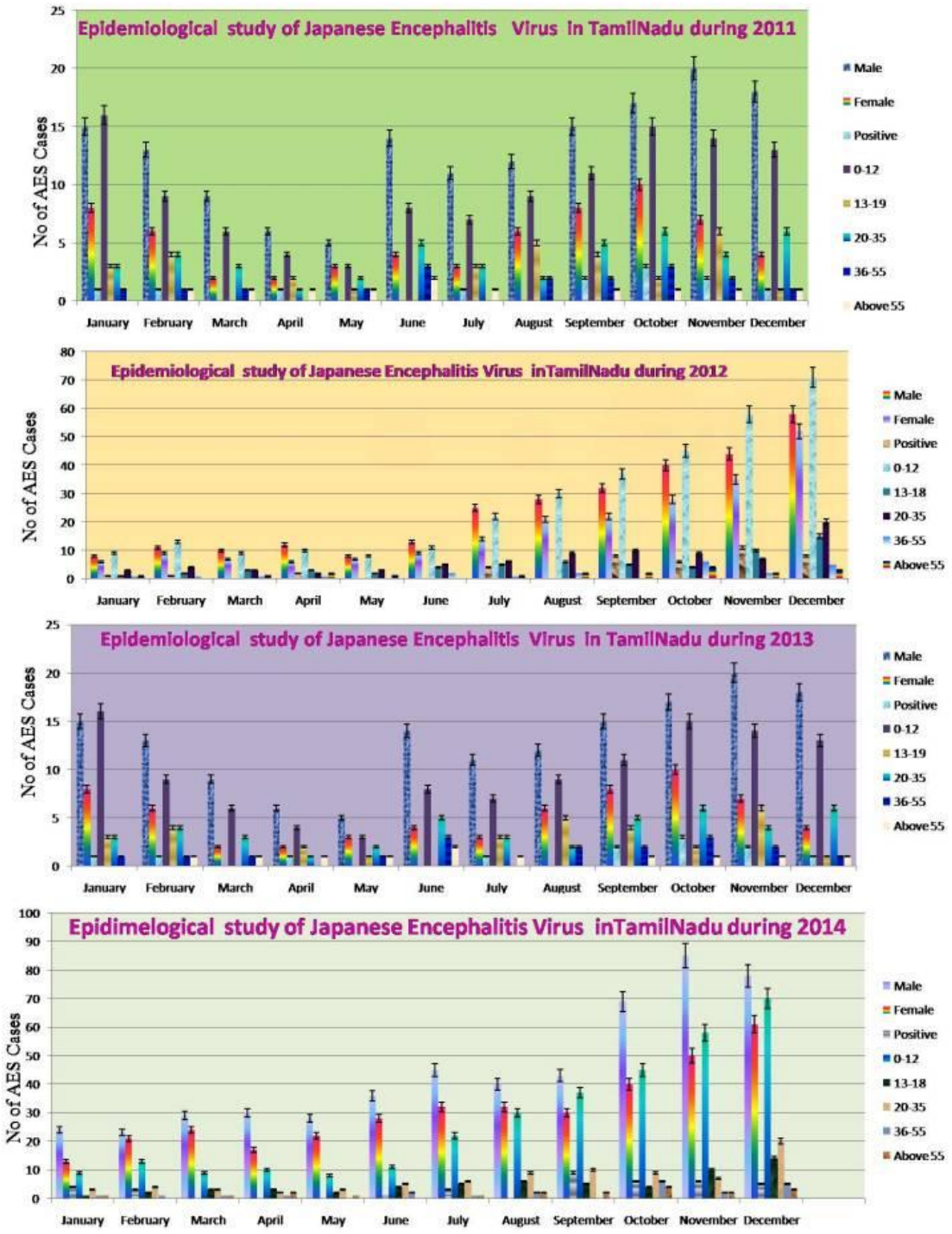


Figure 6.3: Epidemiological study of Dengue virus during the year 2011-2014.

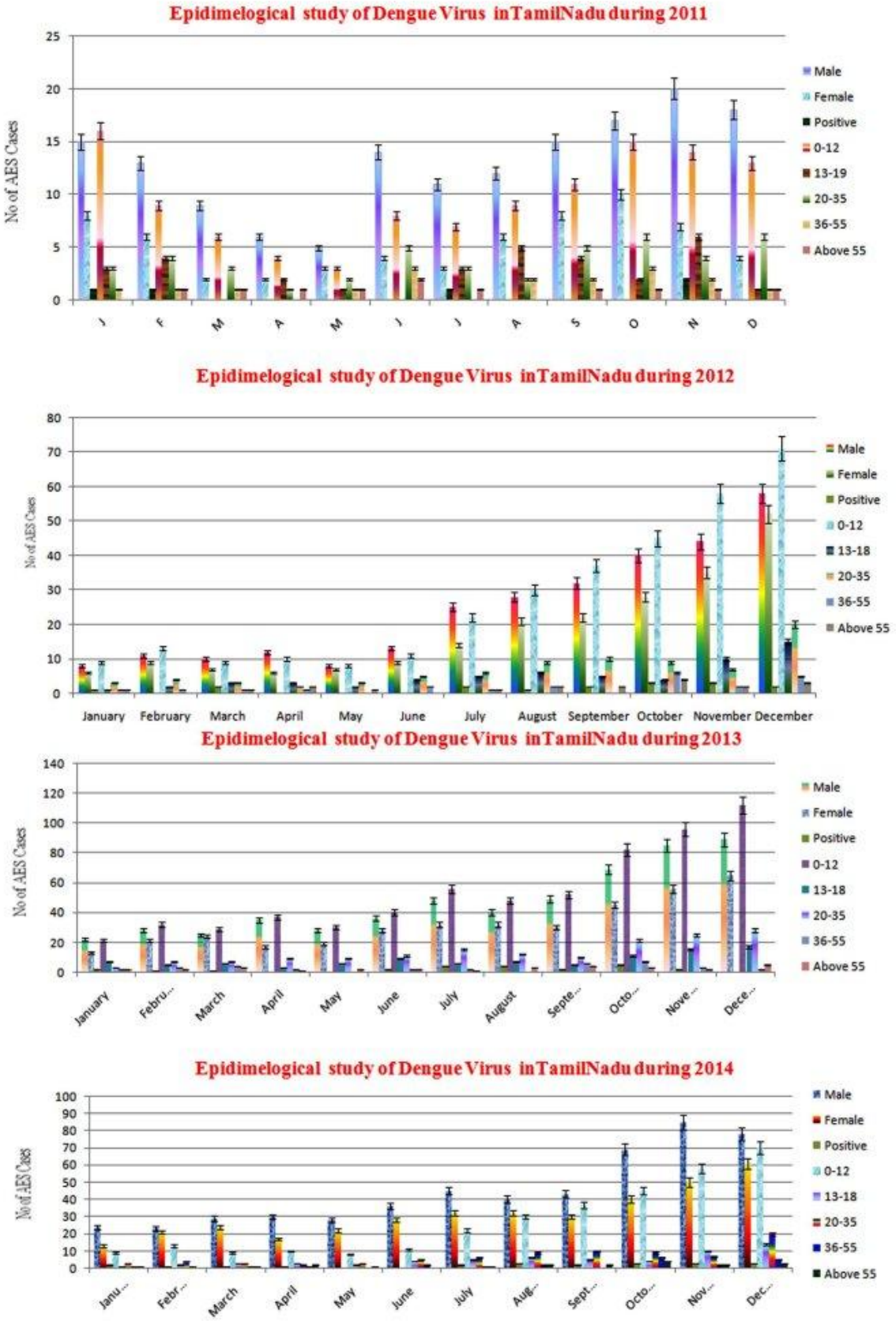
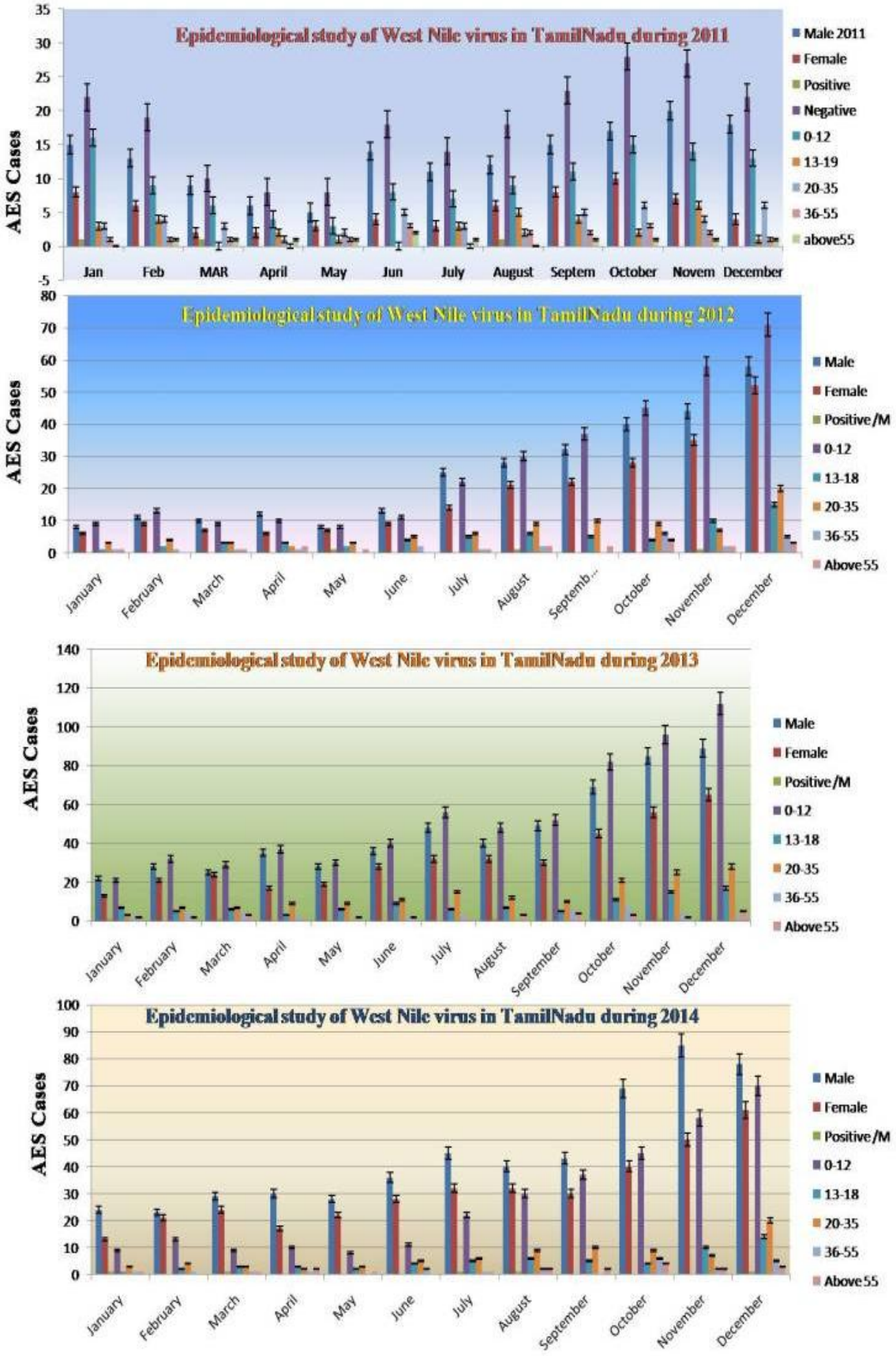


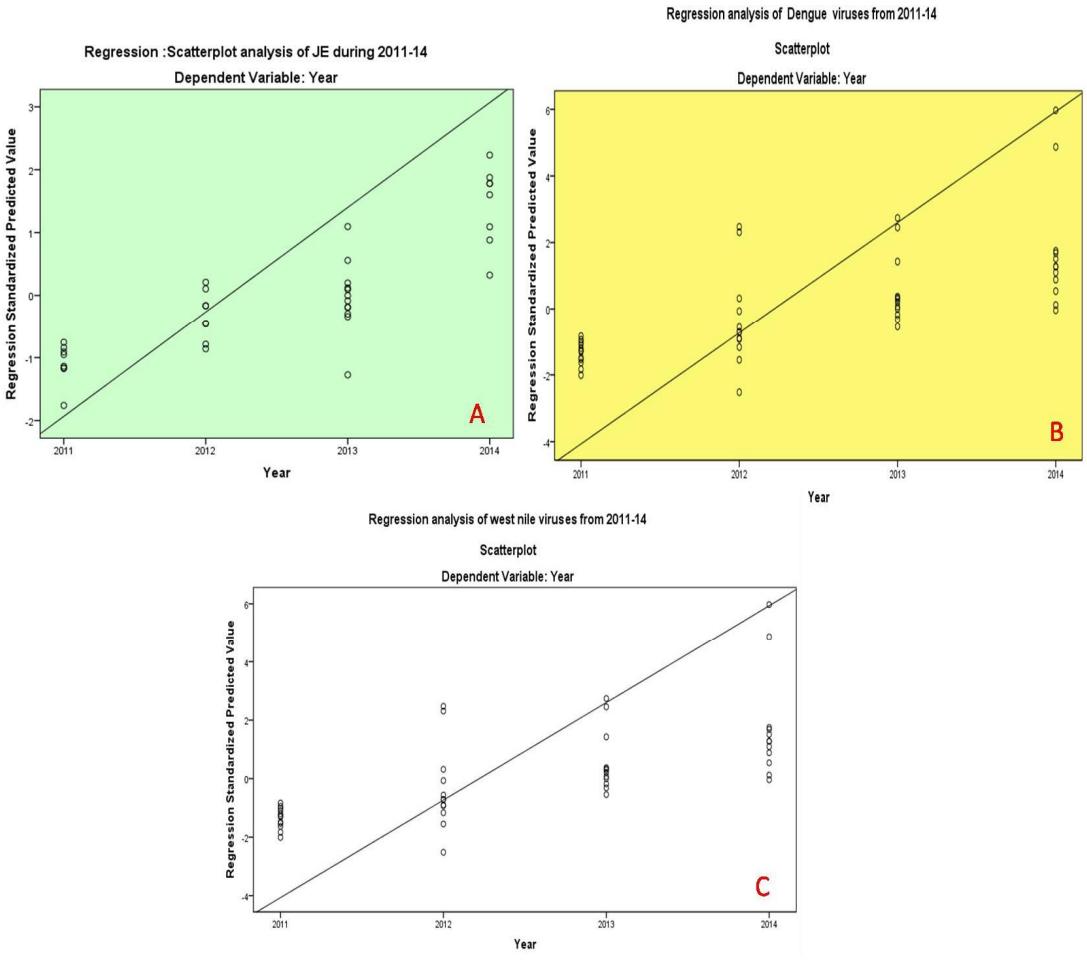
Figure.6.4. Epidemiological study of WNV during the year 2011-2014



The total suspected cases were statistically analyzed using linear regression in different periods from 2011 to 2014. The linear regression in the figure 6.5 suggested that there is an increase in sample size and the total number of suspected cases linearly raised from 2011 to 2014. In comparison of three viruses in AES cases showed that high number of samples were received for Dengue. The positivity of all the flaviviruses in AES cases the study period 2011-14 was in the order of JEV > Dengue >West Nile viruses.

But the regression scatter plot in the figure 6.5. A - C showed that there was an increase in the number of cases for all flaviviral infections though there was not much change in the percentage positivity. The multiple comparison analysis of the study period infers the higher number of positivity was observed in 2013 than 2014.

Figure 6.5: Linear Regression analysis of Positive caes of JE, Dengue and West Nile viruses from 2011-2014



The Post hoc Dunnet statistical analysis inferred that there was significant difference within the group and there was no significant difference between the groups Table. 6.6 to 6.11. The one way ANOVA analysis of different age groups of (JEV, DENV and WNV) cases were analyzed in different periods of the year showed in the table 6.6, 6.8, 6.10. The mean difference was significant in all the age groups with degrees of freedom 0.05 level.

Table 6.6: Statistical Analysis (ANOVA) of Japanese Encephalitis virus during 2011-2014

Dependent Variable		Mean Square	F	Sig.
Male	Between Groups	3101.833	9.636	.000
	Within Groups	321.890		
Female	Between Groups	1880.799	11.412	.000
	Within Groups	164.809		
Positives	Between Groups	22.083	2.807	.055
	Within Groups	7.867		
Negatives	Between Groups	9896.806	10.753	.000
	Within Groups	920.352		
zeroto12yr	Between Groups	3832.132	8.636	.000
	Within Groups	443.737		
Thirteen to 19yrs	Between Groups	61.076	4.810	.006
	Within Groups	12.699		
Twenty to 35yrs	Between Groups	187.910	6.732	.001
	Within Groups	27.911		
Thirty six to 55yrs	Between Groups	3.806	1.245	.305
	Within Groups	3.057		
Above 55yrs	Between Groups	5.076	4.678	.006
	Within Groups	1.085		

* The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table 6.7: Post hoc Dunnet Analysis of Japanese Encephalitis virus during 2011-14

Dependent Variable		Std. Error	Sig.
Male	Dunnett t (2-sided)[a] 2011-12	7.32450	.000
	2012-13	7.32450	.024
	2013-14	7.32450	.986
Female	Dunnett t (2-sided)[a] 2011-12	5.24100	.000
	2012-13	5.24100	.048
	2013-14	5.24100	.995
Positives	Dunnett t (2-sided)[a] 2011-12	1.40243	.082
	2012-13	1.40243	.968
	2013-14	1.28023	.999
Negatives	Dunnett t (2-sided)[a]2011-12	12.38515	.000
	2012-13	12.38515	.030
	2013-14	12.38515	.983
zeroto12yr	Dunnett t (2-sided)[a]2011-12	8.59977	.126
	2012-13	8.59977	1.000
	2013-14	8.59977	.011
Thirteen to 19yrs	Dunnett t (2-sided)[a] 2011-12	1.45481	.267
	2012-13	1.45481	1.000
	2013-14	1.45481	.089
Twenty to 35yrs	Dunnett t (2-sided)[a]2011-12	2.15681	.355
	2012-13	2.15681	1.000
	2013-14	2.15681	.014
Thirty six to5 5yrs	Dunnett t (2-sided)[a]2011-12	.71377	.888
	2012-13	.71377	1.000
	2013-14	.71377	.439
Above 55yrs	Dunnett t (2-sided)[a] 2011-12	.42529	.284
	2012-13	.42529	1.000
	2013-14	.42529	.093

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table 6.8: Statistical Analysis (ANOVA) of Dengue virus during 2011-14.

Variables		Mean Square	F	Sig.
Male	Between Groups	3101.833	9.636	.000
	Within Groups	321.890		
	Total			
Female	Between Groups	1880.799	11.412	.000
	Within Groups	164.809		
	Total			
Positives	Between Groups	1.612	1.562	.221
	Within Groups	1.032		
	Total			
Negatives	Between Groups	9896.806	10.753	.000
	Within Groups	920.352		
	Total			
zeroto12yr	Between Groups	3832.132	8.636	.000
	Within Groups	443.737		
	Total			
Thirteen to19yrs	Between Groups	61.076	4.810	.006
	Within Groups	12.699		
	Total			
Twenty to 35yrs	Between Groups	187.910	6.732	.001
	Within Groups	27.911		
	Total			
Thirty six to 55yrs	Between Groups	3.806	1.245	.305
	Within Groups	3.057		
	Total			
Above 55yrs	Between Groups	5.076	4.678	.006
	Within Groups	1.085		
	Total			

*. The mean difference is significant at the 0.05 level

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table 6.9: Post hoc Dunnet Analysis of Dengue virus during 2011-14

Dependent Variable		Std. Error	Sig.
Male	Dunnett t (2-sided)[a] 2011-12	7.32450	.000
	2012-13	7.32450	.024
	2013-14	7.32450	.986
Female	Dunnett t (2-sided)[a] 2011-12	5.24100	.000
	2012-13	5.24100	.048
	2013-14	5.24100	.995
Positives	Dunnett t (2-sided)[a] 2011-12	.60109	.387
	2012-13	.48194	.994
	2013-14	.46683	.667
Negatives	Dunnett t (2-sided)[a] 2011-12	12.38515	.000
	2012-13	12.38515	.030
	2013-14	12.38515	.983
Zero to 12 yr	Dunnett t (2-sided)[a] 2011-12	8.59977	.126
	2012-13	8.59977	1.000
	2013-14	8.59977	.011
Thirteen to 19 yrs	Dunnett t (2-sided)[a] 2011-12	1.45481	.267
	2012-13	1.45481	1.000
	2013-14	1.45481	.089
Twenty to 35 yrs	Dunnett t (2-sided)[a] 2011-12	2.15681	.355
	2012-13	2.15681	1.000
	2013-14	2.15681	.014
Thirty six to 55 yrs	Dunnett t (2-sided)[a] 2011-12	.71377	.888
	2012-13	.71377	1.000
	2013-14	.71377	.439
Above 55 yrs	Dunnett t (2-sided)[a] 2011-12	.42529	.284
	2012-13	.42529	1.000
	2013-14	.42529	.093

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table 6.10: Statistical Analysis (ANOVA) of West Nile virus during 2011-14

Variables		Mean Square	F
Male	Between Groups	3101.833	9.636
	Within Groups	321.890	
	Total		
Female	Between Groups	1880.799	11.412
	Within Groups	164.809	
	Total		
Positives	Between Groups	.000	.
	Within Groups	.000	
	Total		
Negatives	Between Groups	9896.806	10.753
	Within Groups	920.352	
	Total		
zeroto12yr	Between Groups	3832.132	8.636
	Within Groups	443.737	
	Total		
Thirteento19yrs	Between Groups	61.076	4.810
	Within Groups	12.699	
	Total		
Twentyto35yrs	Between Groups	187.910	6.732
	Within Groups	27.911	
	Total		
thirtysixto55yrs	Between Groups	3.806	1.245
	Within Groups	3.057	
	Total		
Above55yrs	Between Groups	5.076	4.678
	Within Groups	1.085	
	Total		

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table 6.11: Post hoc Dunnet Analysis of West Nile virus during 2011-14

Dependent Variable		Std. Error	Sig.
Male	Dunnett t (2-sided)[a]	7.32450	.000
	2011-12	7.32450	.024
	2012-13	7.32450	.986
	2013-14		
Female	Dunnett t (2-sided)[a]	5.24100	.000
	2011-12	5.24100	.048
	2012-13	5.24100	.995
	2013-14		
Negatives	Dunnett t (2-sided)[a]	12.38515	.000
	2011-12	12.38515	.030
	2012-13	12.38515	.983
	2013-14		
zeroto12yr	Dunnett t (2-sided)[a]	8.59977	.126
	2011-12	8.59977	1.000
	2012-13	8.59977	.011
	2013-14		
Thirteento19yrs	Dunnett t (2-sided)[a]	1.45481	.267
	2011-12	1.45481	1.000
	2012-13	1.45481	.089
	2013-14		
Twentyto35yrs	Dunnett t (2-sided)[a]	2.15681	.355
	2011-12	2.15681	1.000
	2012-13	2.15681	.014
	2013-14		
Thirty six to55yrs	Dunnett t (2-sided)[a]	.71377	.888
	2011-12	.71377	1.000
	2012-13	.71377	.439
	2013-14		
Above55yrs	Dunnett t (2-sided)[a]	.42529	.284
	2011-12	.42529	1.000
	2012-13	.42529	.093
	2013-14		

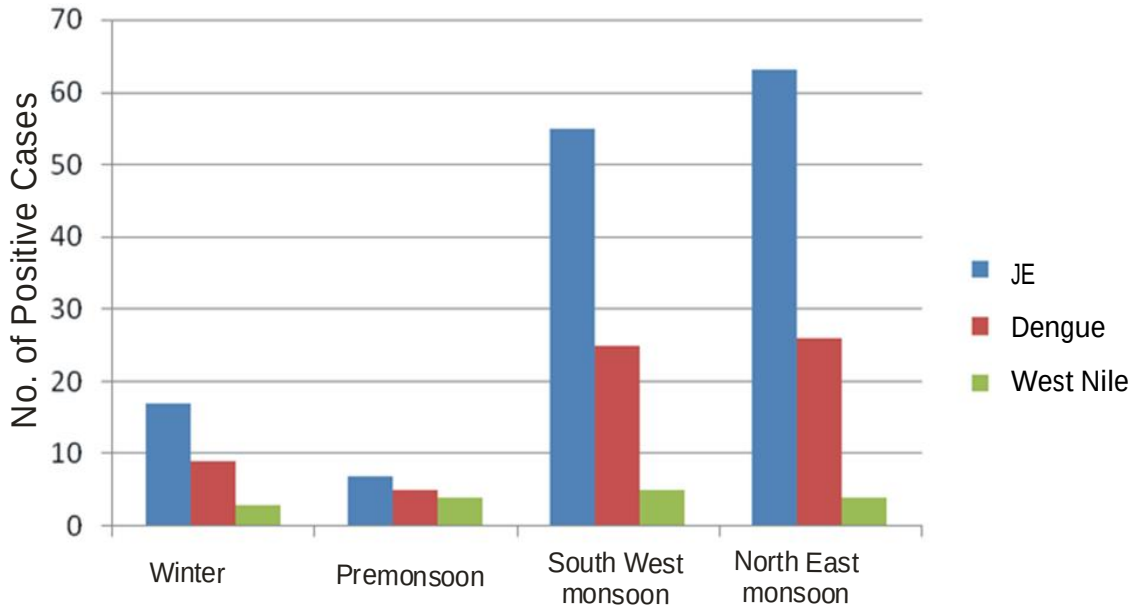
*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

6.4 SEASON WISE DISTRIBUTION OF AES CASES

The season wise distribution of cases was statistically analyzed for their epidemiological characterization of the AES. The inference of the seasons of AES susceptibility was represented in the figure 6.6. Among the three flaviviruses, the highest positive cases were due to Japanese Encephalitis viral infection in all the seasons except pre monsoon. The Dengue viruses have highest positive cases in the South West and North East monsoon seasons. Susceptible cases were found to be lower during the winter and pre monsoon than other seasons. The positive cases due to West Nile viral infection were higher in the South West monsoon than other seasons. On the whole, the JE viral infection was found to be frequent during the study period 2011-14 when compared to Dengue and West Nile viral infections.

Figure 6.6: Season wise distribution of JE, Dengue and West Nile virus from 2011-2014.



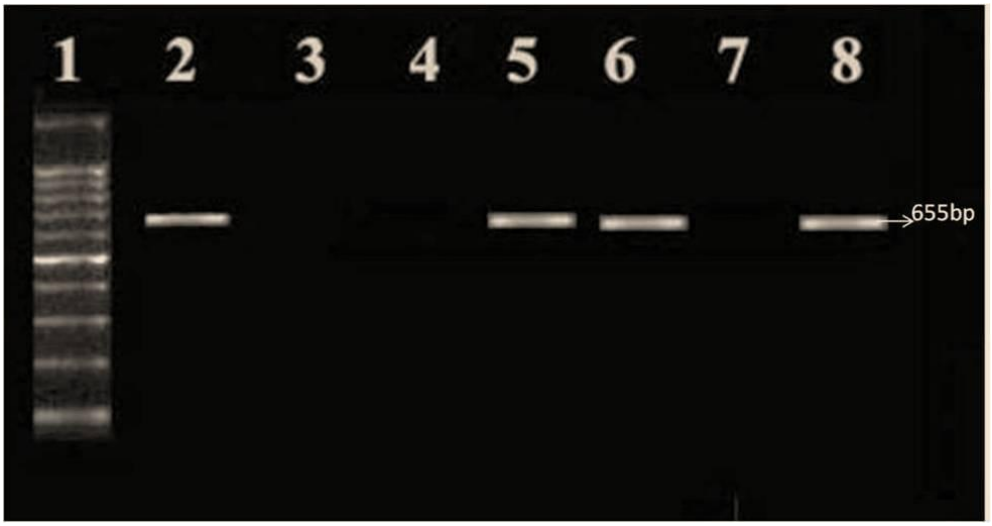
6.5 GENDER WISE DISTRIBUTION OF JE, DENGUE AND WEST NILE VIRUSES

The gender wise distribution of positive cases were statistically analyzed for Japanese Encephalitis, Dengue and West Nile viruses. The month wise and gender wise analysis were done and represented in the figure 6.2 to 6.4. The susceptible cases were frequently observed in the month of October, November and December during the study period. The positive cases of flaviviruses were low in the 2011 and 2014 figure 6.1. Among the three falaviviruses, the Japanese Encephalitis cases occurred more than the West Nile and Dengue viruses. Gender wise analysis indicated that positives and susceptible cases were high in males than females.

6.6 MOLECULAR CHARACTERIZATION OF JE, DENGUE AND WEST NILE VIRUSES

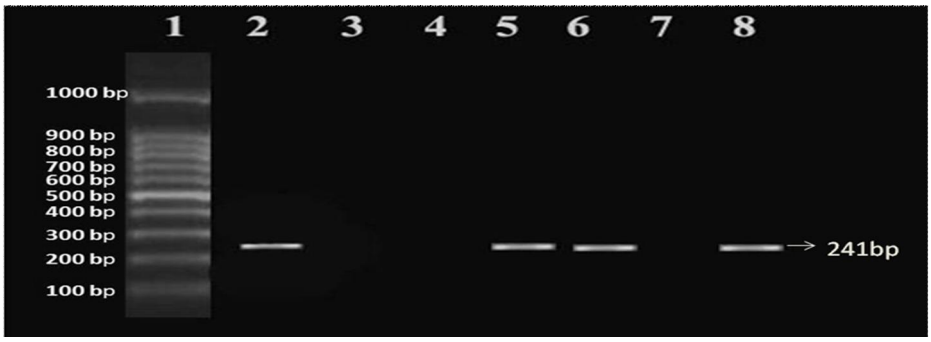
Totally, 324 CSF samples were subjected to Pan-flavi conventional polymerase chain reaction (PCR). Among them, 24 samples were confirmed as positive for Pan-flavi PCR characterized by the amplified product of 655bp (Figure 6.6).

Figure 6.6: Agarose gel analysis of Pan-flavi RT-PCR. Lane 1. Molecular weight marker; Lane 2 to Lane 6 samples; Lane 7. Negative control; Lane 8.Positive control



Further, the positive samples were amplified using specific primers for Japanese Encephalitis (JEV) characterized by the amplified product of 241bp (Figure 6.7).

Figure 6.7: Agarose gel analysis of JEV RT-PCR. Lane 1. Molecular weight marker; Lane 2 to Lane 6 samples; Lane 7. Negative control; Lane 8.Positive control



Dengue positives were first subjected to Pan Dengue characterized by the amplified product of 511bp then it was further segregated for D3 and D4. The Dengue (DENV-3) was characterized by the amplified product of 288bp (Figure 6.8), D4 260bp (Figure 6.9) and West Nile viruses (WNV) characterized by the amplified product of 396bp (Figure 6.10).

Figure 6.8: Agarose gel analysis of Pan Dengue RT-PCR. Lane 1. Molecular weight marker; Lane 2 to Lane 6 samples; Lane 7. Negative control; Lane 8.Positive control

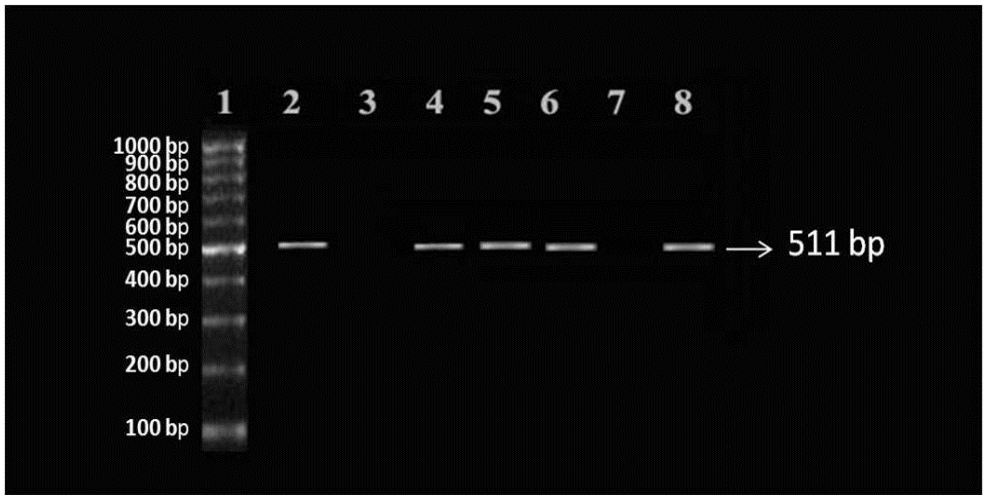


Figure 6.9: Agarose gel analysis of Pan Dengue-3 & 4 RT-PCR lane. 1. Molecular weight marker;Lane 2 to Lane 6 samples; Lane 7. Negative control; Lane.8. Positive control

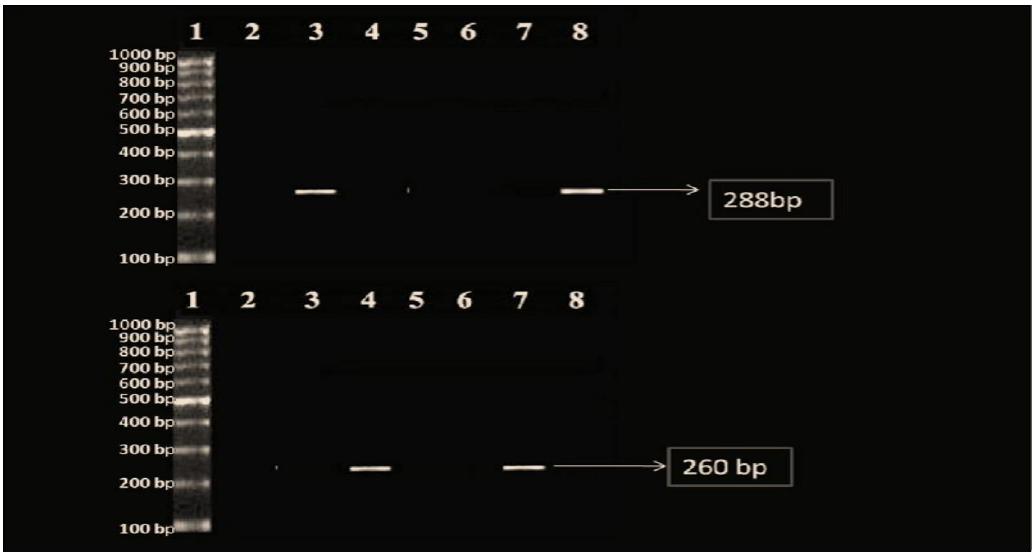
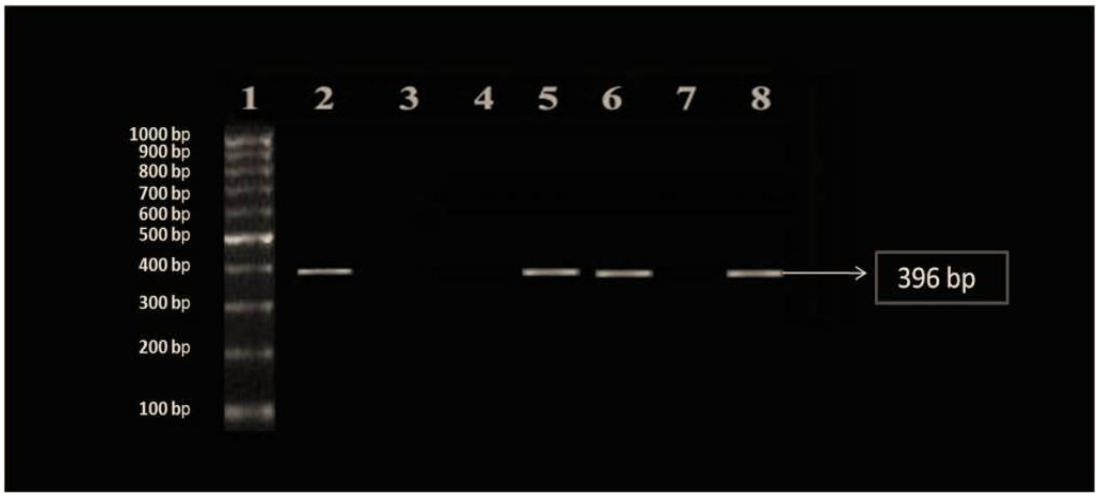


Figure 6.10: Agarose gel analysis of West Nile RT-PCR. Lane 1. Molecular weight marker; Lane 2 to Lane 6 samples; Lane 7. Negative control; Lane 8.Positive control



Of the 24 positive cases, 15, 6 and 3 samples were positive for JEV, DENV and WNV respectively Table 6.11. More number of CSF cases was occurred in the years 2013 and 2014. Finally the positive samples were subjected to sequence analysis for further molecular characterization. During the study period, the Japanese Encephalitis virus was found to be the most predominant flavivirus among the three flaviviruses. The positive CSF cases of all flaviviruses were predominant among the age group of pediatric and adolescent.

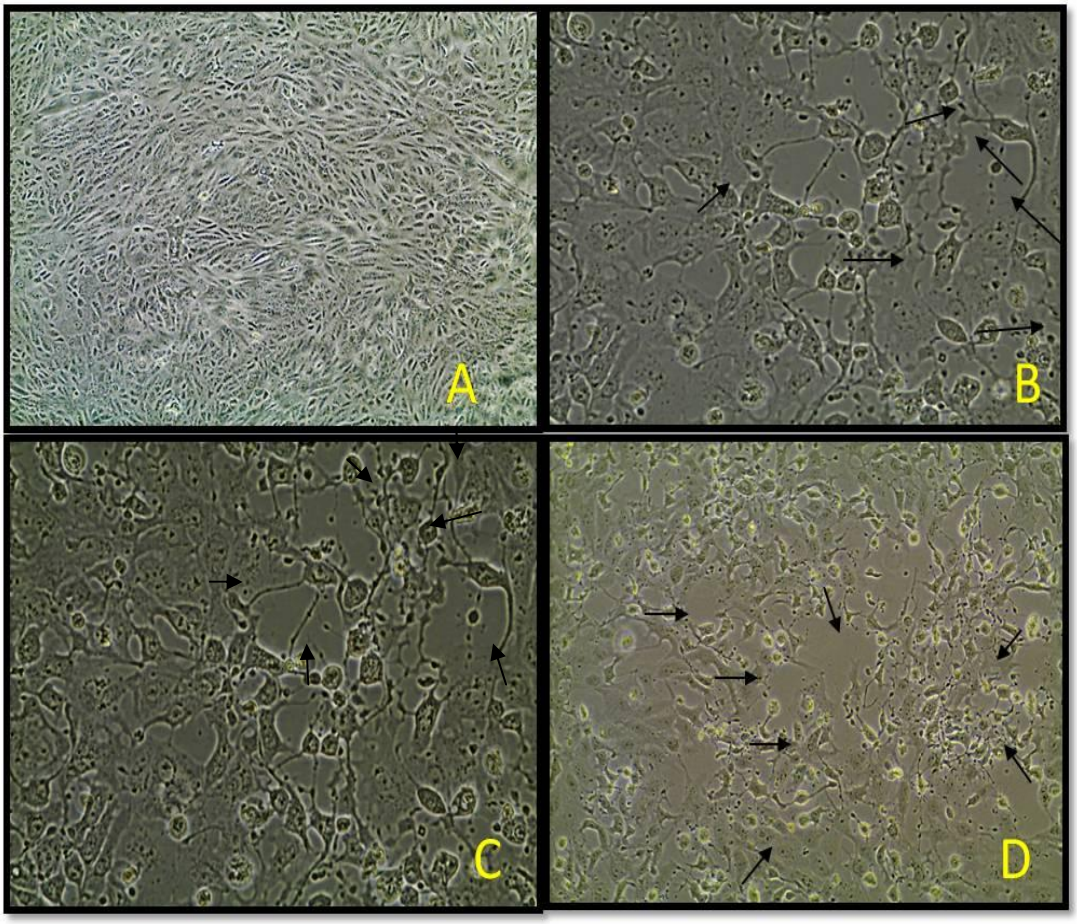
Table 6.11: Year wise positive results of PCR in CSF

Year	Total samples	Positive	JE Positive	Dengue Positive	WNV Positive
2011	24	0	0	0	0
2012	65	8	5	2	1
2013	111	12	8	3	1
2014	124	4	2	1	1

6.7 VIRUS ISOLATION AND CHARACTERIZATION BY HAEMAGGLUTINATION

The PCR positive samples were subjected to virus isolation using Vero cell lines. The PCR positives of the flaviviruses (JEV, DENV, WNV) showing cytopathic effect were represented in the figure 6.11.

Figure 6.11: Cytopathic effect of JEV, DENV and WNV in Vero cell line A: Control, B: JE infected cells, C: Dengue infected cells, D: West Nile infected cells



The isolated JE virus was more compatible in the early passages of 2 and 4, rest of the passages were less sensitive and less specific. The Dengue virus was more compatibly isolated in the late 6th passage. West Nile virus was isolated from the infected Vero cells up to 5th passage of cell culture. The virally infected cells exhibited CPE representing the changes in cellular architecture such as cell rounding, shrinkage and enlargement between the cells expresses as gaps in the figure 6.11B, C & D and the virus infected cells were then subjected to haemagglutination in different pH (6, 6.2 and 6.4). The optimum pH for the haem-agglutination of the flaviviruses were represented in the figure 6.12. The optimum pH of Haem-Agglutination in JE virus was optimally agglutinated at pH 6.2, shown in the figure 6.12 Similarly, the Dengue and West Nile viruses agglutinated well at

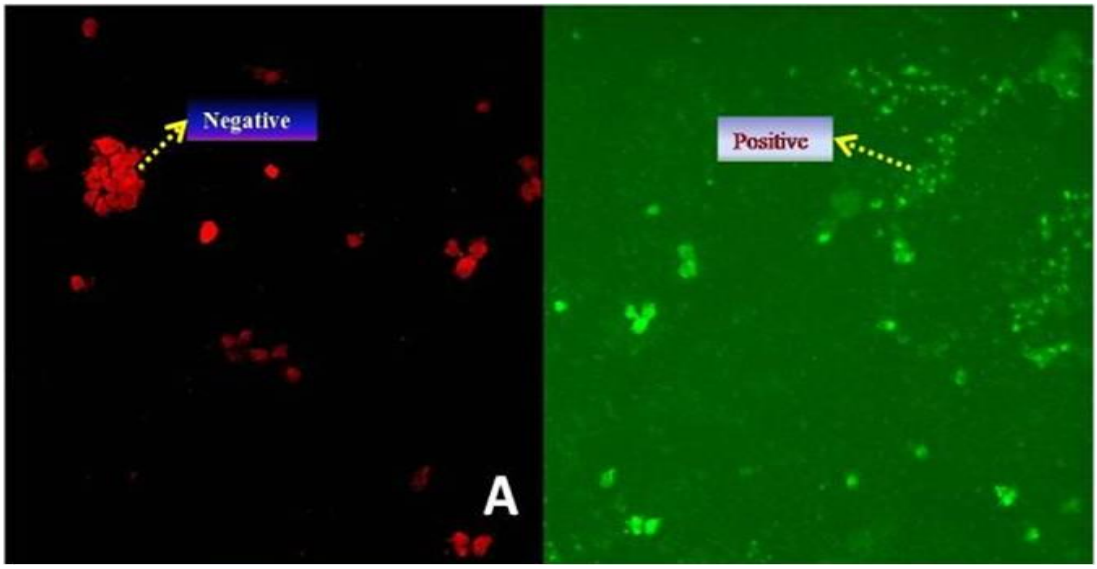
the optimum pH of 6.4. The viral titre of JE viruses was optimally observed as 1:128. Similarly the Dengue viruses showed the optimum titre of 1:256 and West Nile virus 1:32.

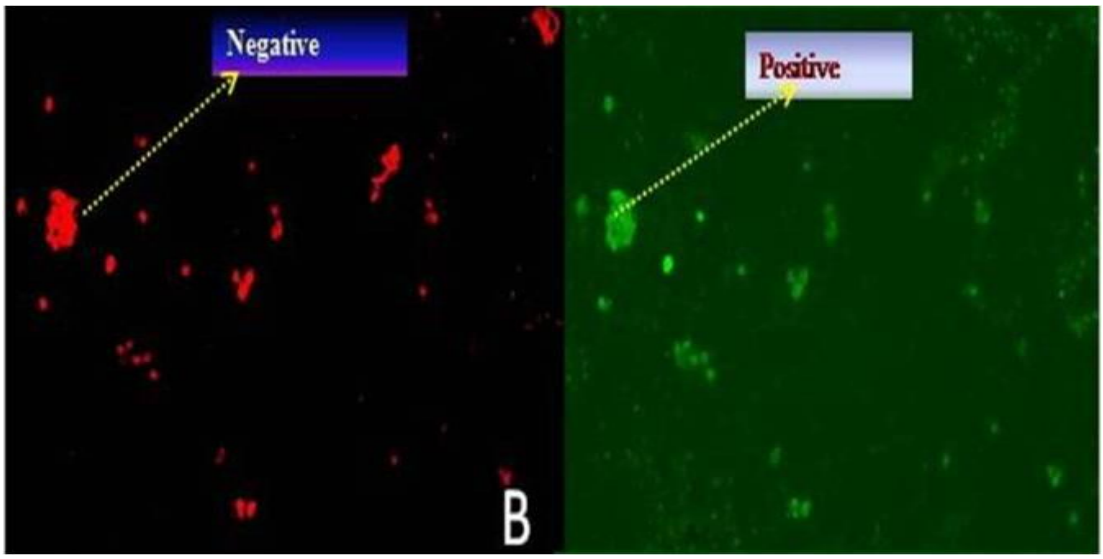
Figure 6.12: Haemagglutination assay.A: JEV, B: DENV, C: WNV

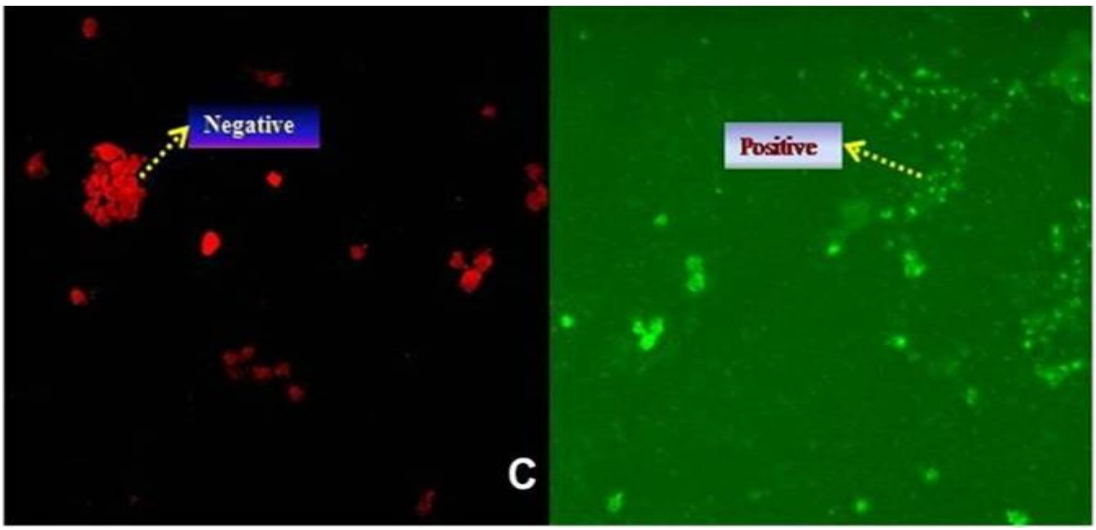
6.8 IMMUNOFLUORESCENCE ASSAY FOR JE, DENGUE AND WEST NILE VIRUS

Further characterization of flaviviruses were carried out by Immunofluorescence Assay (IFA). The IFA results of the viral infection of cells were represented in the figure 6.13. The figure shows the determination of positive and negative by the subsequent appearance of virus by antihuman IgM antibody fluorescent tag. The JE positive IFA was clearly appeared as apple green colour. The uninfected cells was observed as cherry red colour due to the non adherent binding of anti human IgM antibody fluorescent tag shown in figure 13A. Similarly the Immunofluorescence Assay (IFA) of Dengue virus was showh in figure 13B and West Nile virus displayed in figure 13C. The dilution of 1:10 stains good fluorescence intensity with less noise in the background.

Figure 6.13: Immunofluorescence Assay. A:JEV, B: DENV, C: WNV







6.9 MOLECULAR PHYLOGENY AND SEQUENCE ANALYSIS OF JAPANESE ENCEPHALITIS VIRUS ISOLATES

The amplicons were purified using QIA quick PCR purification kit (Qaigen) and subjected to cycle sequencing using ABI Prism Big Dye Terminator V3.1 cycle sequencing kit. Post-cycle sequencing purification was performed using Dye Ex2.0 spin kit (Qiagen). Sequencing was performed on ABI Prism 310 and sequence alignment and phylogenetic analyses were done using Mega version 6.

The nucleotide sequencing of JEV were presented in the figure and the sequence was deposited with the accession number from Gene Bank of National Center for Biological Information (NCBI) were assigned and presented in the figure 6.14. The figure 6.15 & 6.16 represents the phylogenetic tree derived from the gene sequences of JEV 1 and 2 along with wild isolates.

The phylogram showed 1 and 2 gene sequences of the isolates [Genbank: KJ947880] belonging to the non-structural protein -5 gene and comprising 98% - 100% nucleotide similarity with each other. The rooted tree of JE current sequence analysis shows that the phylogram have a common ancestor of gene sequence belonging to India gi|496909/India/2005/envelope. The KIPM JE sequences were highly homologous with the clade of Indian sequences 2009 and 2011 gi|239633849/India/2009/ envelope and gi|289466091/India/2011/envelope.

The phylogenetic distances between the two close sequences with the query sequences was calculated in the Mega and found as 0.021. The second close clade of JE/TN/ KIPM sequences have 96% higher similarity with US, China and Japan with accession number of gi|1778238 / USA / 1997 / envelope, gi|410026435/China/2014/envelope and gi|52546172/Japan/2009/ polyprotein shown in the figure.6.16. The phylogenetic distance was calculated in the second clade and it was found to be 0.0148.

Figure 6.14: Sequencing and deposition of Japanese Encephalitis virus isolates in NCBI

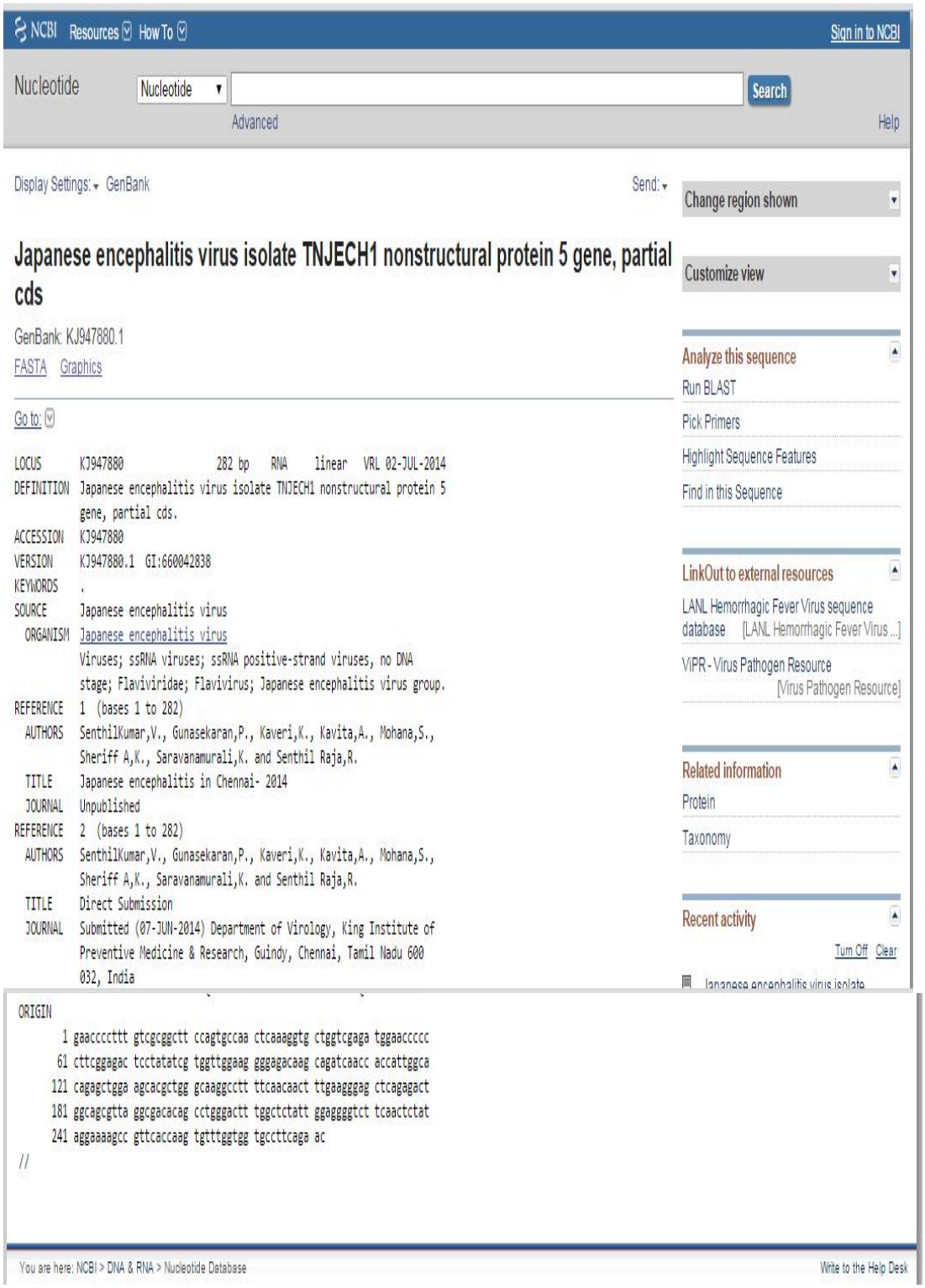


Figure 6.15: Radial phylogenetic tree of Japanese Encephalitis virus

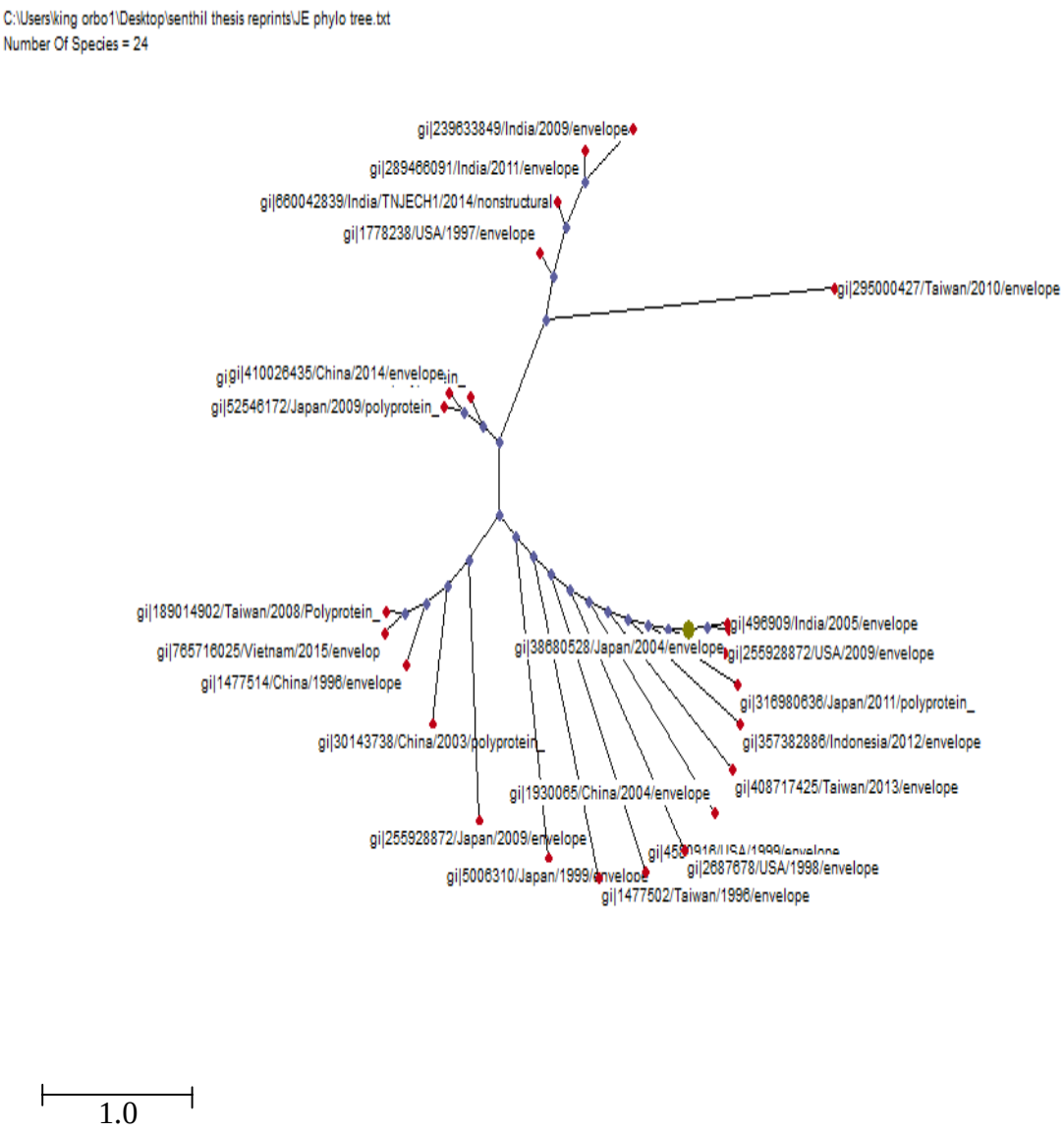
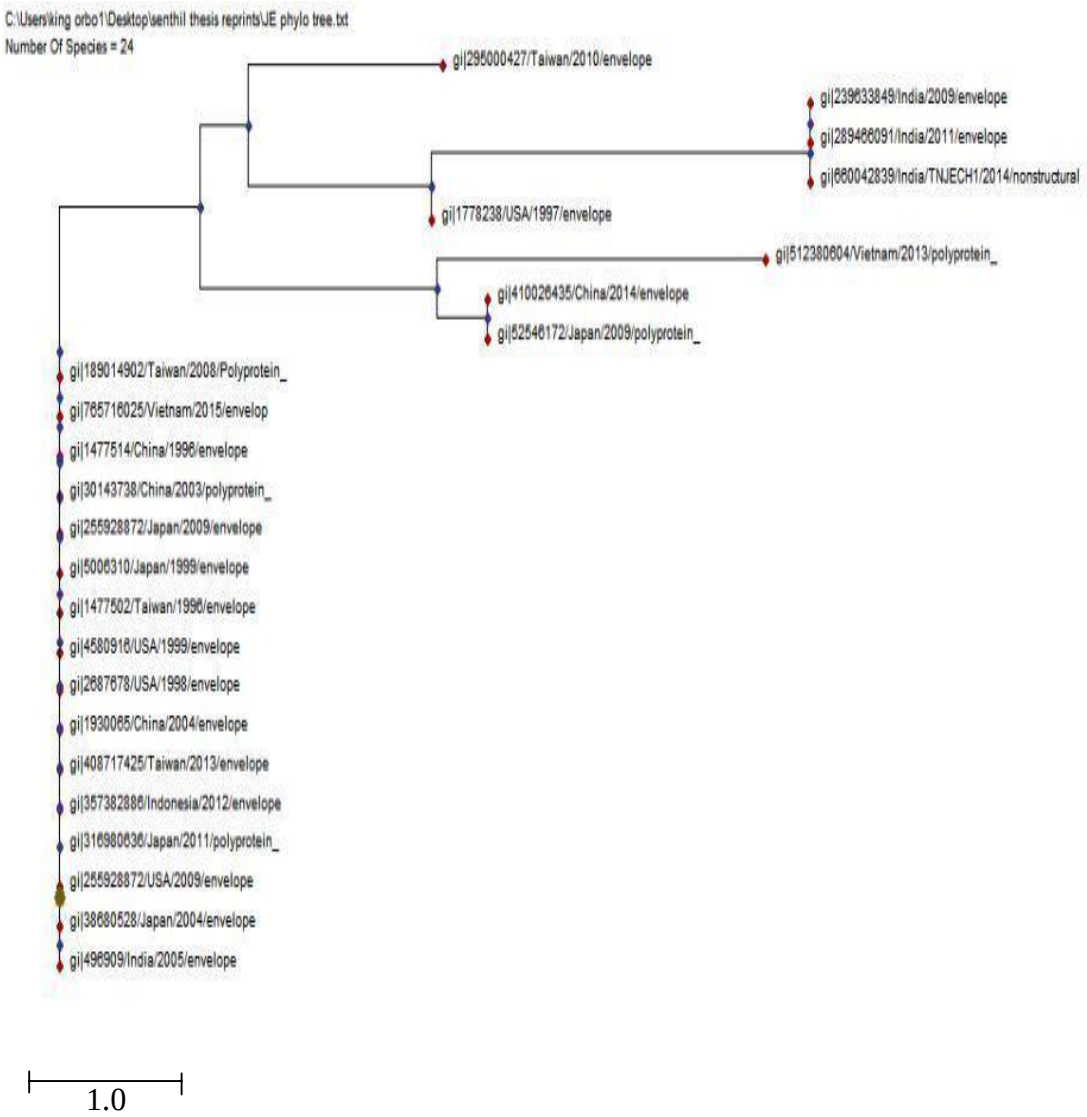


Figure 6.16: Phylogenetic analysis of Japanese Encephalitis virus representing phylogram



6.10 MOLECULAR PHYLOGENY AND SEQUENCE ANALYSIS OF DENGUE-3 AND DENGUE- 4 ISOLATES

The KIPM DENV 3 sequence was submitted in NCBI and accessesion number showed in figure 6.17 & 6.18. It was highly homologous with the clade of Taiwan (gi/ 25992032/Taiwan/2006/Polyprotein) (gi/25992032/Taiwan/2006/Polyprotein), Peru (gi/19071812 peru/2002/capsid) and Sri Lanka (gi 164654854 srilanka/2015/Anchored). The above clades were highly close to the KIPM KJ947880. The phylogenetic distances between the closely related sequences and the query sequence was calculated using Mega and found to be 0.0425 (Figure 6.19 & 6.20).

The second close clade of Dengue KIPM sequences have 96% higher similarity with Brazil and other parts of Indian sequences of Maharashtra (gi|574486451/India/Maharashtra/2014/polyprotein) and Thailand (gi|116874465/ Thailand/2011/polyprotein). The phylogenetic distance was calculated in the second clade and it was found to be 0.0158. Similarly, the Dengue 4 sequences were highly homologous with the clade of Manila (gi|923096874/Manila/2015/polyprotein) and Finland (gi|675895418/ Finland/2014/polyprotein).

The above clades were closely related to the KIPM 732562361 sequences. The phylogenetic distances between the closely related sequences with that of the query sequence was calculated in the Mega and it was found to be 0.0486. The second close clades of Dengue KIPM sequences have 94-96% higher similarity with Brazil (gi|443909555/Brazil/2013/polyprotein), Japan (gi|321117306/Japan/2011/polyprotein) and Germany (gi|5823462/Germany/ 1999/polyprotein). The phylogenetic distance between the groups of sequences in the second clade was found to be 0.0312 (Figure 6.20).

Figure 6.17: Sequencing of Dengue 3 virus isolate and deposition in NCBI

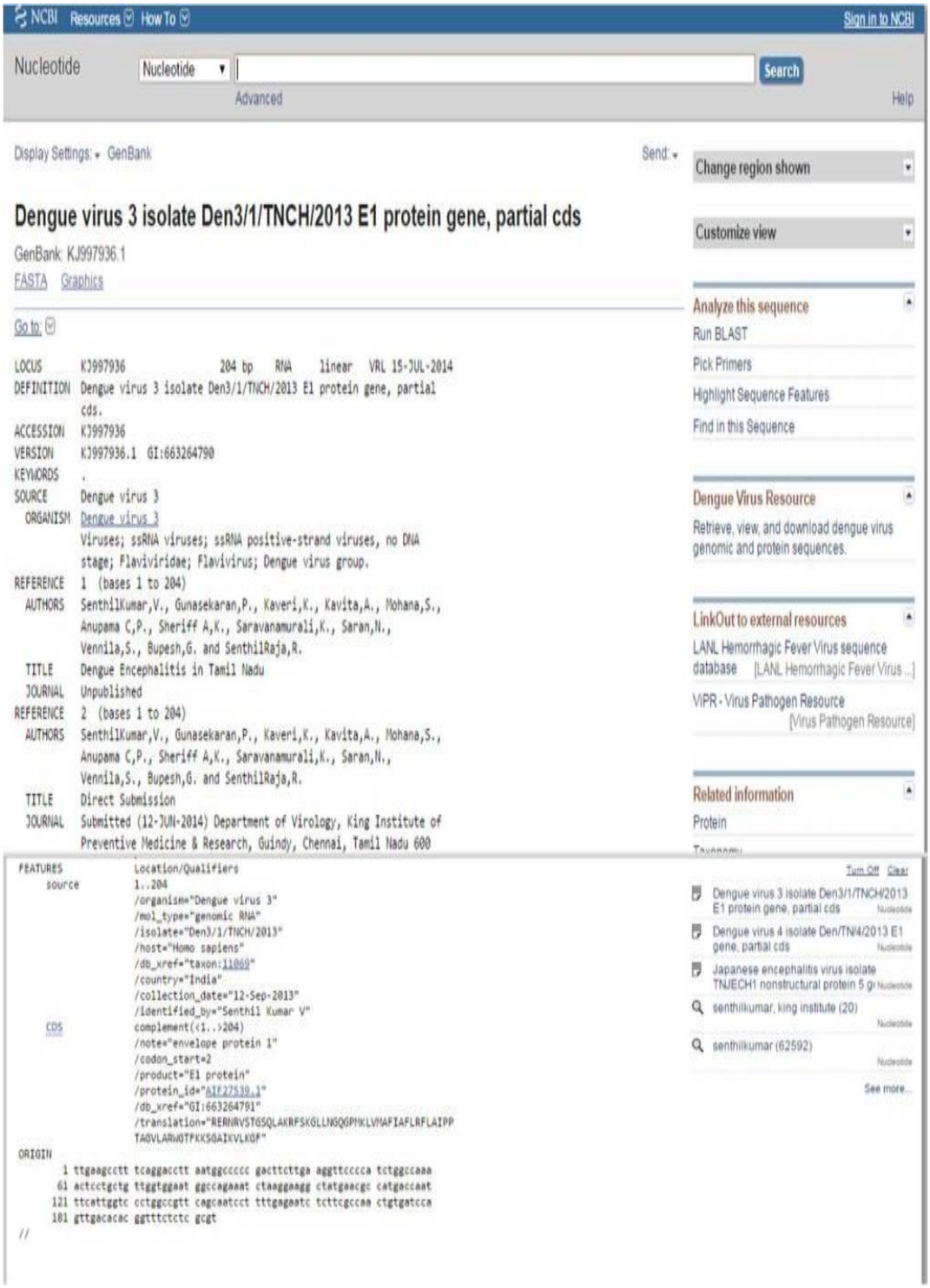


Figure 6.18: Sequencing of Dengue-4 virus isolate and deposition in NCBI

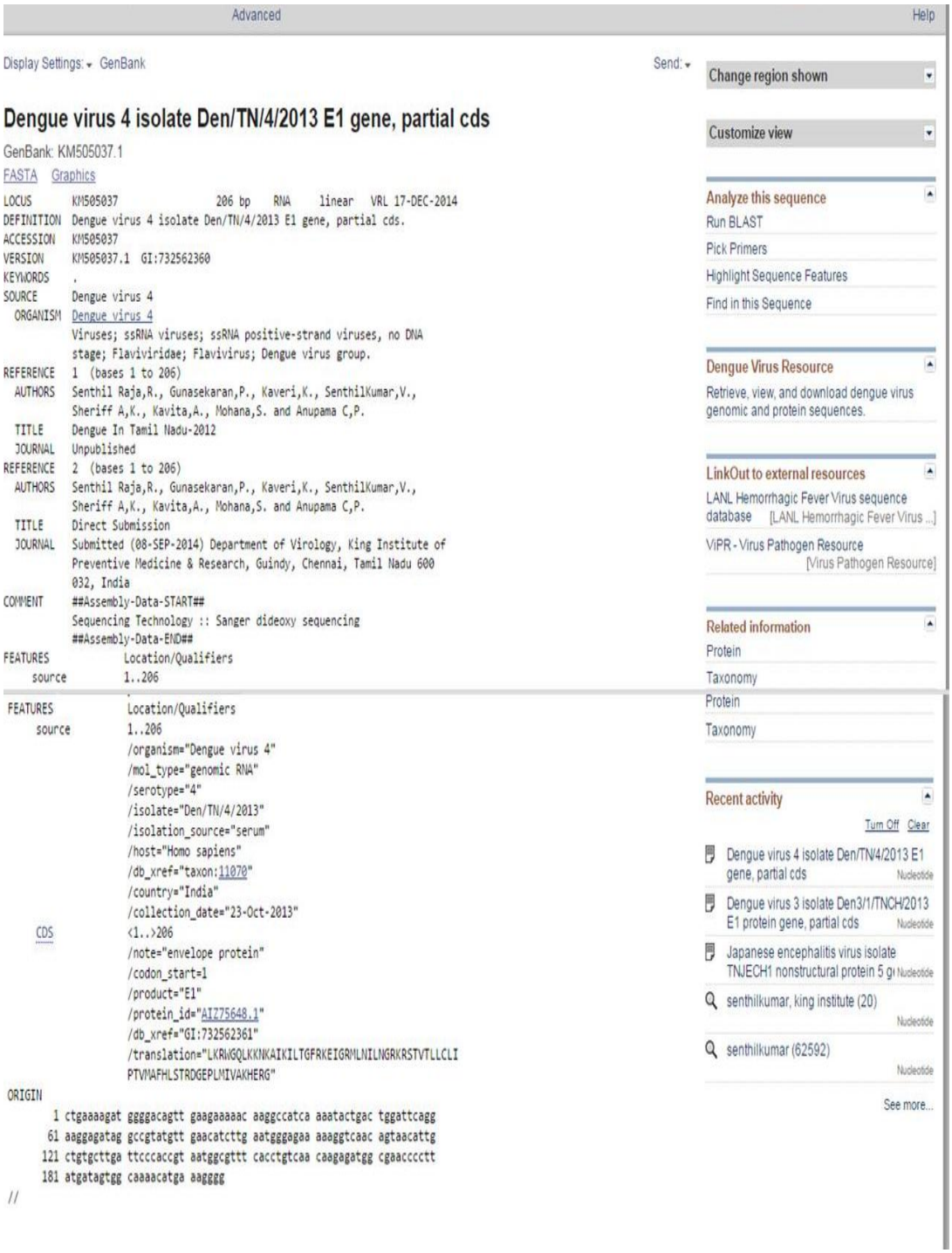


Figure 6.19: Radial phylogenetic tree of Dengue-3 virus isolate

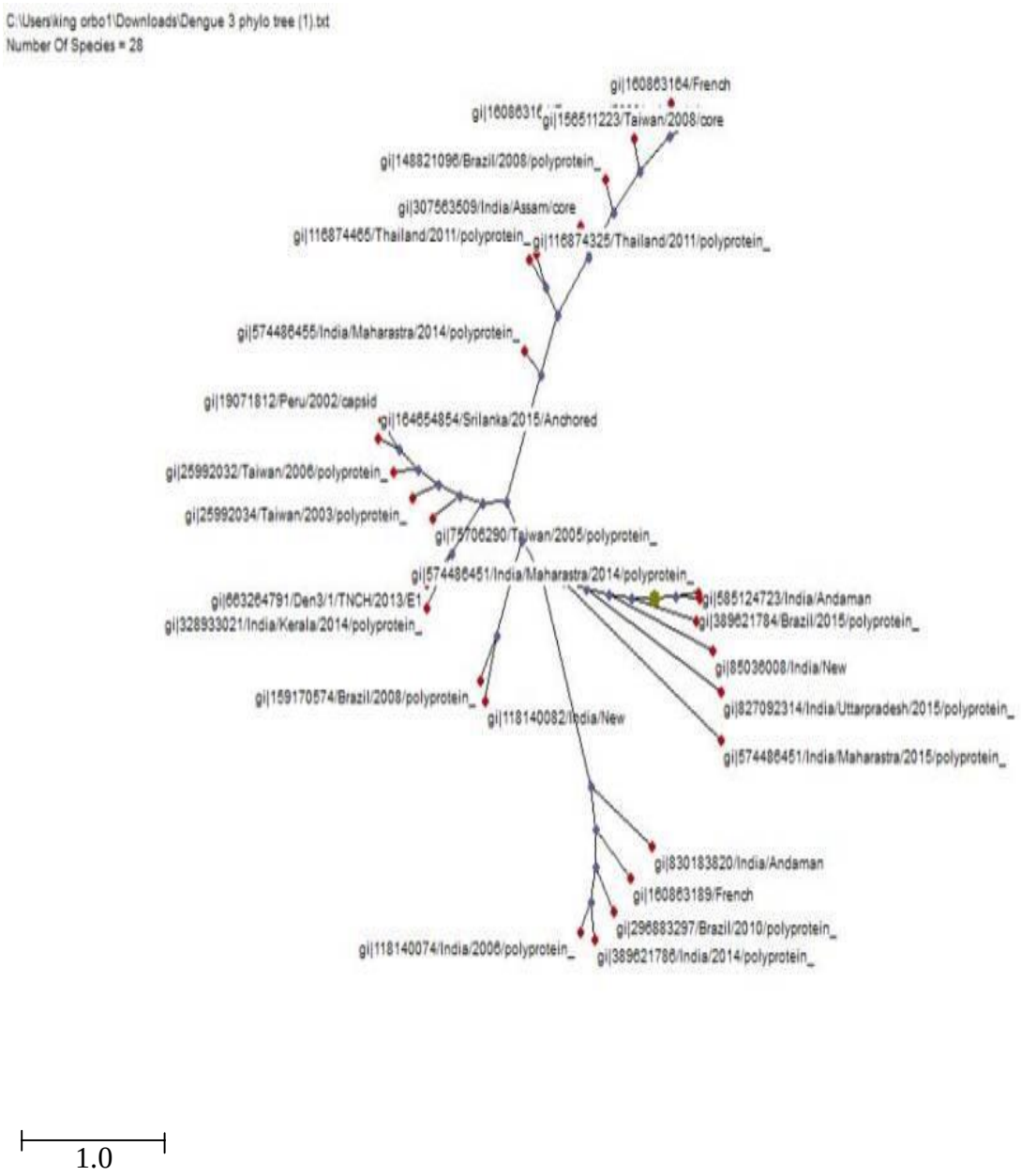
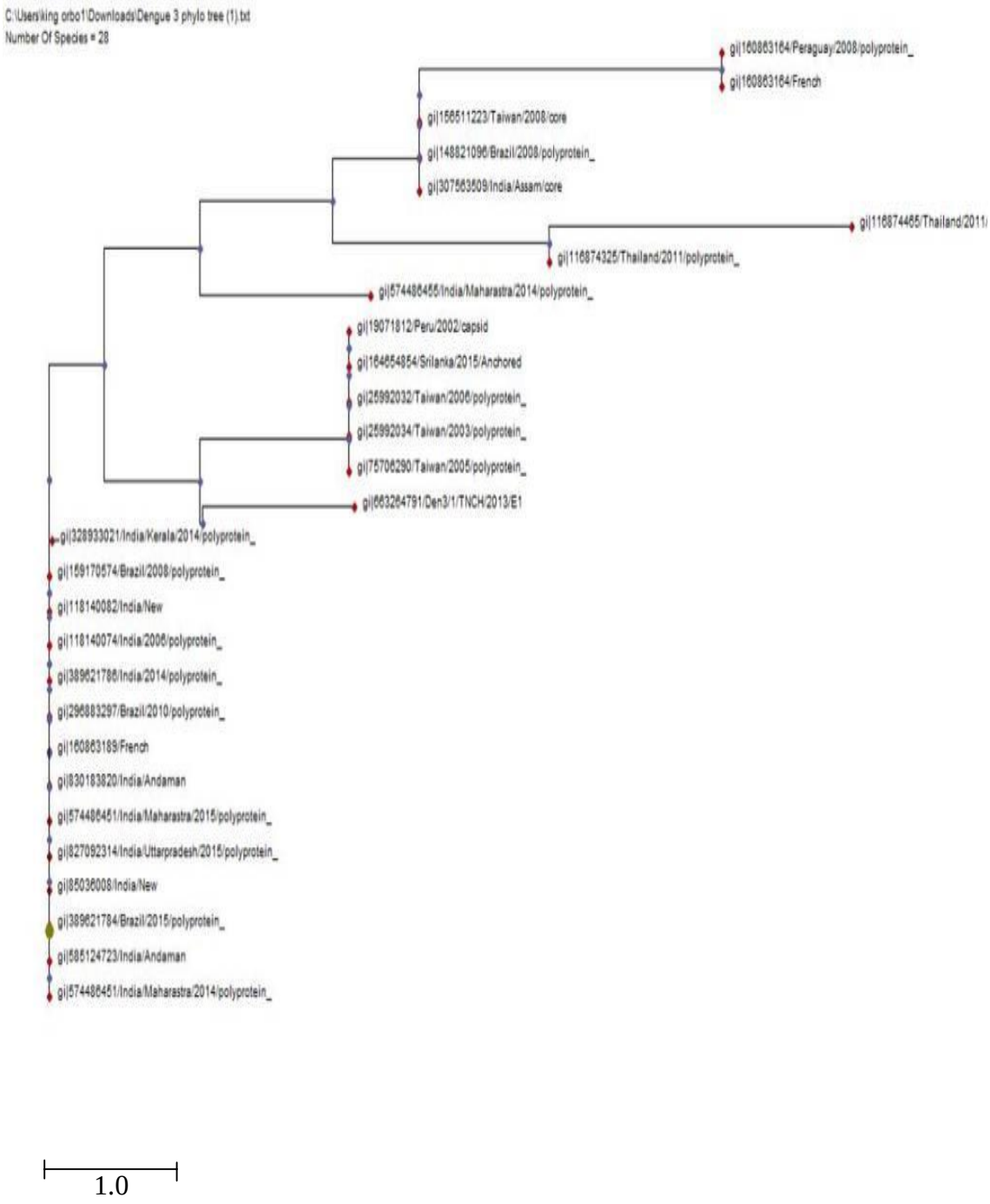


Figure 6.20: Phylogenetic analysis of Dengue-3 representing phylogram



6.11 MOLECULAR PHYLOGENY AND SEQUENCE ANALYSIS OF WEST NILE VIRAL ISOLATES

The PCR amplicons of West Nile viruses were purified using QIAquick PCR purification kit (Qaigen) and subjected to cycle sequencing using ABI Prism Big Dye terminator V3.1 cycle sequencing kit. Post-cycle sequencing purification was done using Dye Ex 2.0 spin kit (Qiagen). WNV Sequencing was performed on ABI Prism 310 and sequence alignment and phylogenetic analyses were done using Mega version 6. The nucleotide sequencing of WNV were presented in the figure.6.21 & 6.22 and the sequence was deposited with the accession number from Gene Bank of NCBI.

The figures 6.23 & 6.24 represents the phylogenetic tree derived from the gene sequences of WNV 1 and 2 along with wild isolates. The phylogram showed 1 and 2 gene sequences of the isolates [Genbank: KP170483 and KP170484] belonging to the Polyprotein gene and comprising 98%-100% nucleotide similarity with each other. The rooted tree of WNV current sequence analysis shows that the phylogram have the common ancestor of gene sequence belonging to USA Polyprotein (gi|260533850/USA/2009/polyprotein).

The KIPM WNV sequences were highly homologous with the clade of Madagascar (gi|58618099/ Madagascar/2006/polyprotein) and USA (gi|27735298/ USA/2012/unnamed) shown in the figure 6.23. The phylogenetic distances of sequences with the query sequence was calculated using the Mega and it was found to be 0.021. The second close clade of WNV/TN/ KIPM sequences have 96% higher similarity with Israel (gi|290246783/Israel/2011/polyprotein) and USA gi|158516889/USA/2015/ unnamed) shown in the figure 6.23 of radial tree. The phylogenetic distance was calculated in the second clade and it was found to be 0.0133.

Figure 6.21: Sequencing of West Nile virus isolate and deposition in NCBI

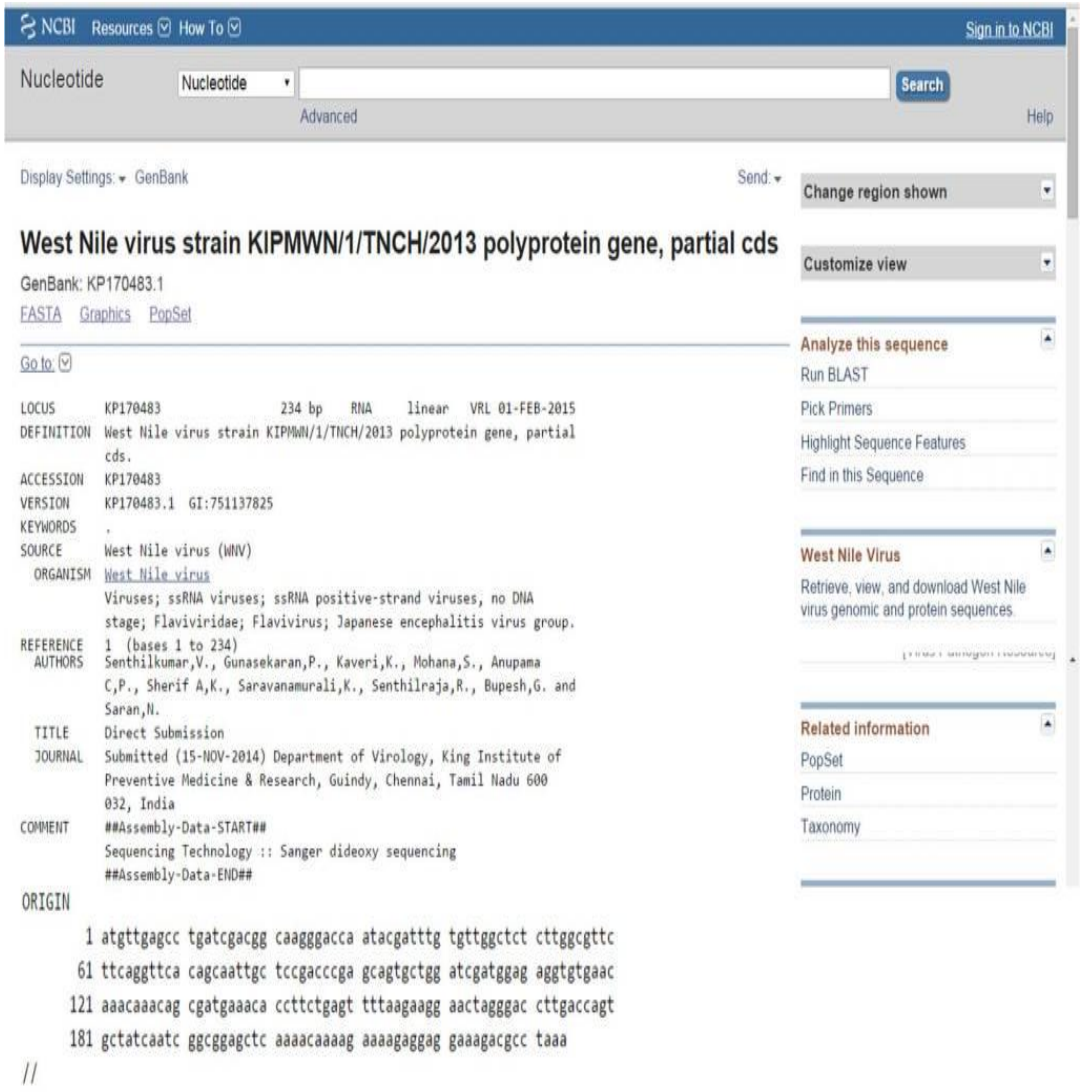


Figure 6.22: Sequencing of West Nile virus isolate and deposition in NCBI

Figure 6.23: Phylogenetic analysis of West Nile virus representing phylogram

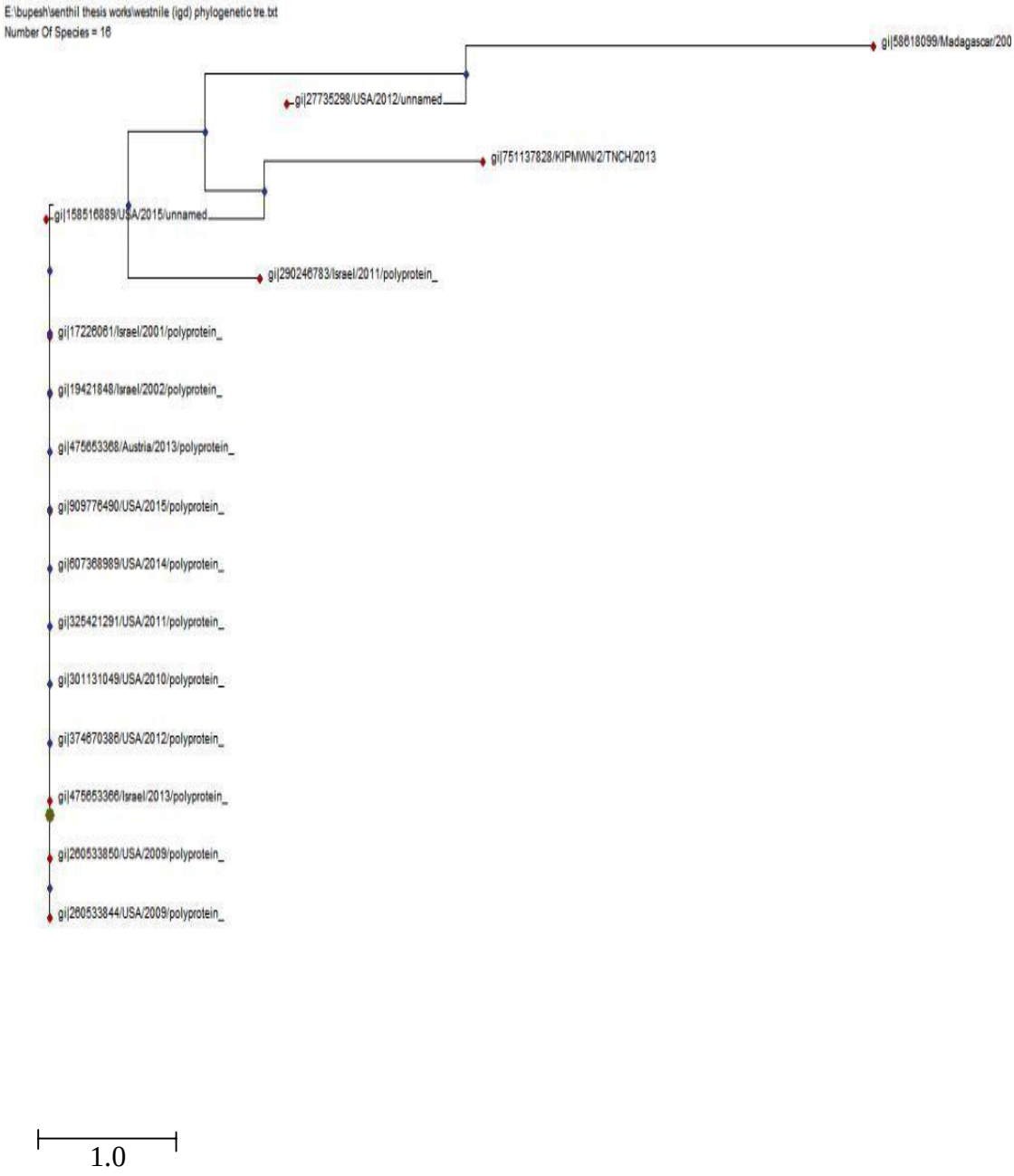
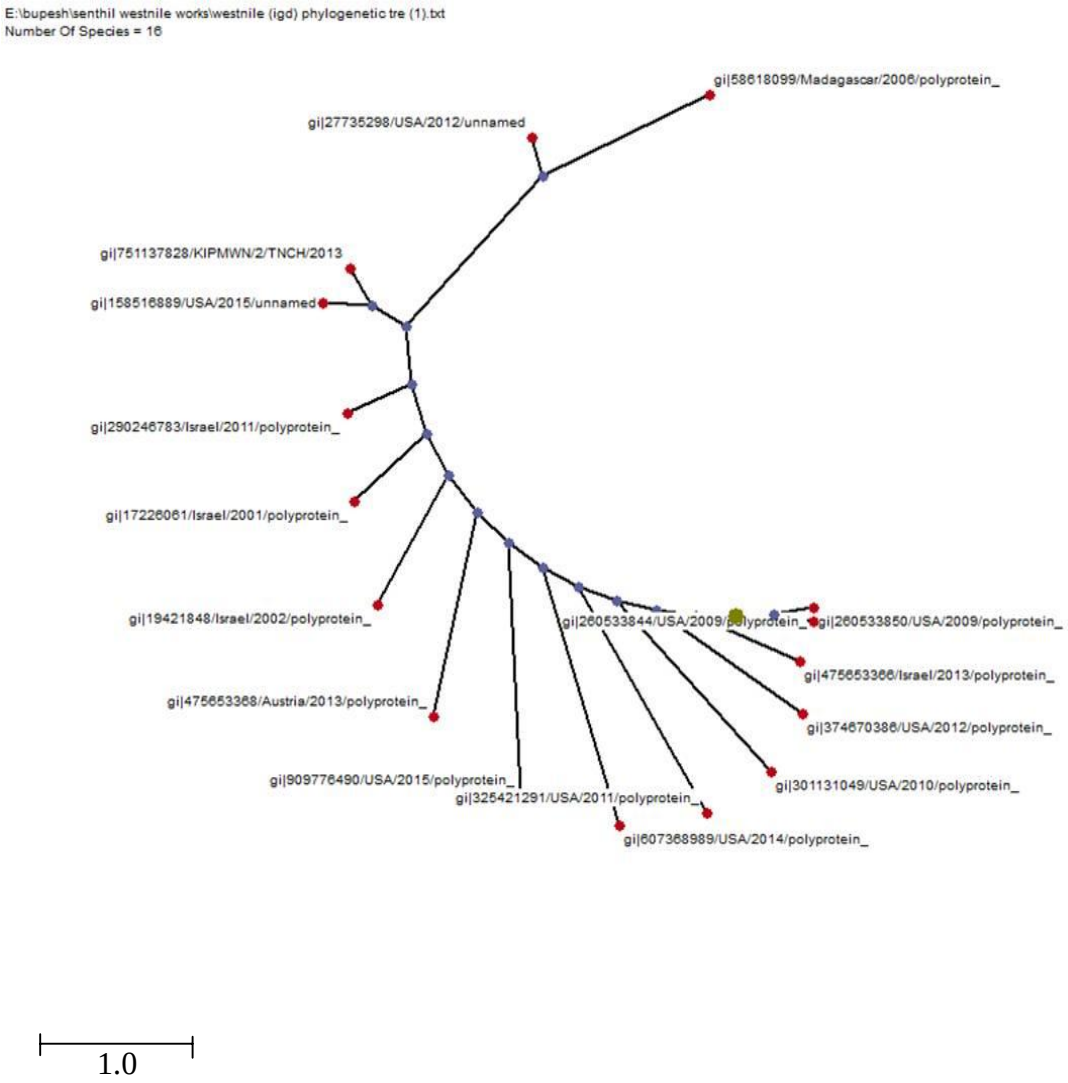


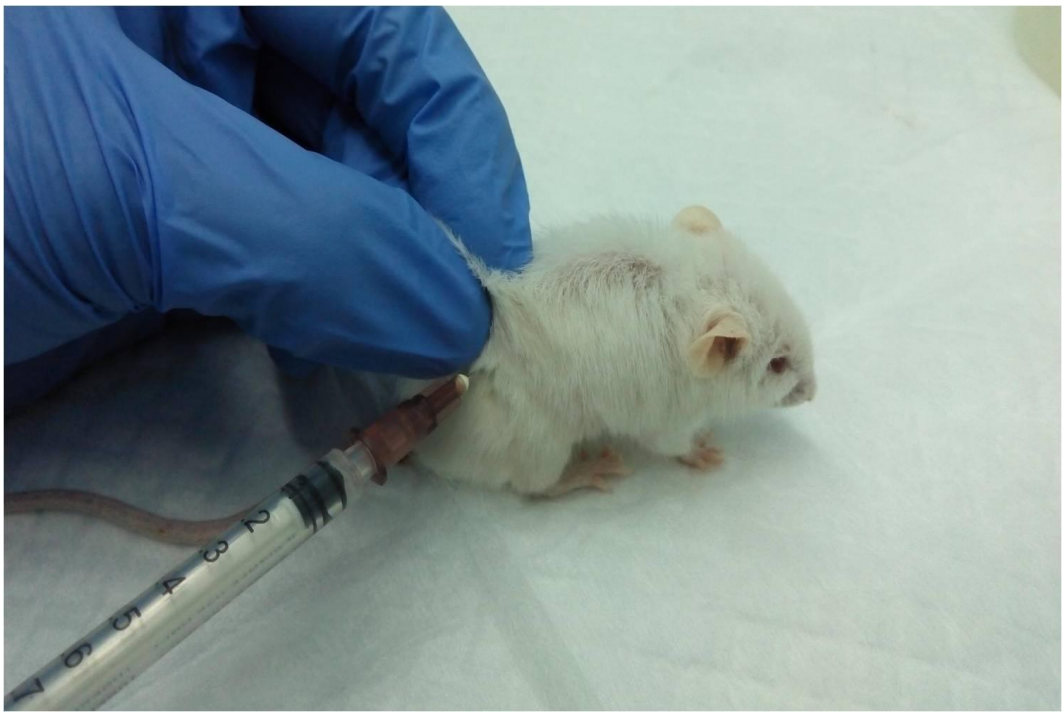
Figure 6.24: Radial phylogenetic tree of West Nile virus isolate



6.12 DEVELOPMENT AND STANDARDIZATION OF PAN-FLAVI DETECTION SYSTEM IN SWISS ALBINO MICE MODEL

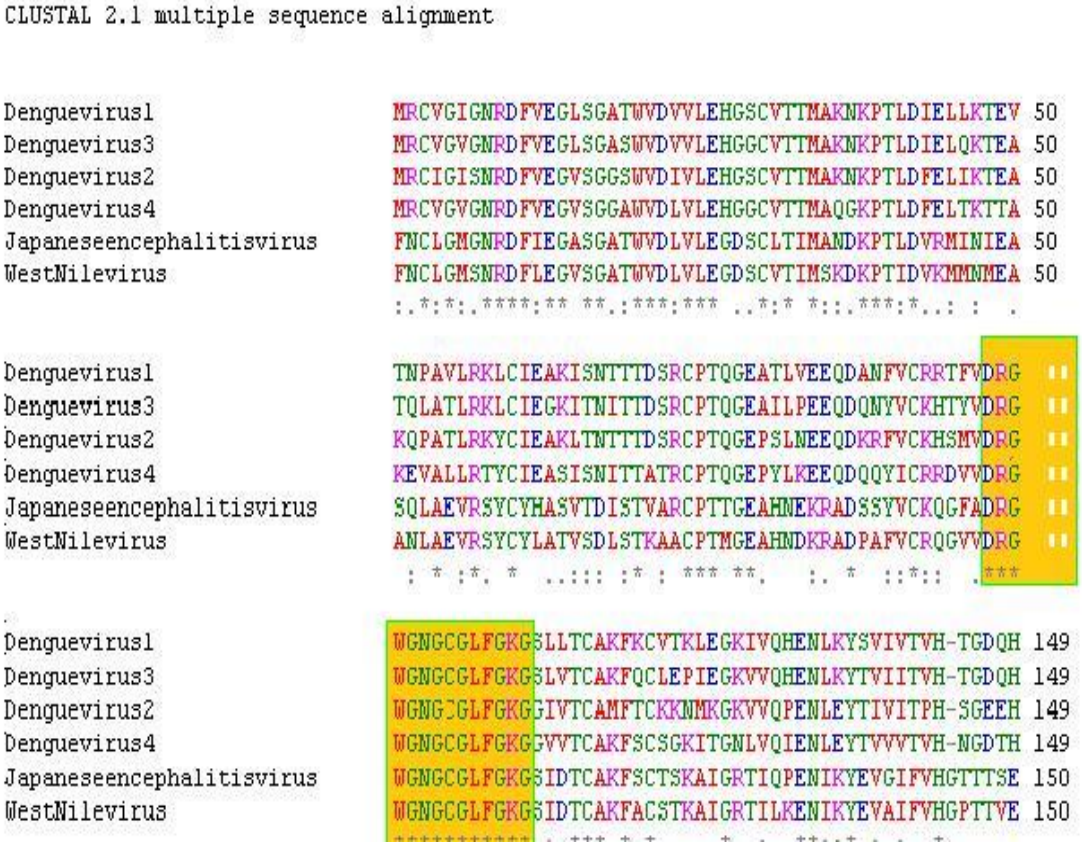
The common conserved sequences of pan-flaviviruses were evaluated for the toxicity and immune response to develop diagnosis for viruses in mice model represented in the figure 6.25.

Figure 6.25: Intramuscular Administration of Fusogenic peptide in Swiss albino mice



Similarly, in the mice model the synthetic peptide of pan-flavi common conserved sequences (Figure 6.27) of DENV, JEV and WNV were mined using the Clustal W tools and designed for peptide synthesis.

Figure 6.26: Multiple sequence alignment of flaviviruses by Clustal W tool



Then, the designed fusogenic peptide was evaluated *in vivo* through the Swiss Albino mice model. The mice were initially administered 100µg intramuscularly in 21 days old mice with 20g body weight shown in the figure 6.25. The LD_{50} was found to be 100µg. Further, the effective concentration was calculated and it was found to be 50µg. All the treated groups were sacrificed in the end of the booster dose of synthetic peptide administered as per the standard immunization protocol. Then the liver, spleen, heart and kidney were subjected for the histopathological observation presented in the figure 6.27. The liver treated with the synthetic fusion peptide and control were compared and represented in the figure 6.27A. The control liver displays the normal cellular architecture. But in the fusion peptide treated liver showed the massive necrosis and alteration in the hepatocytes shown in the figure 6.27B.

Figure 6.27: Histopathological study of Fusogenic peptide (from flavivirus) in swiss albino mice

A: Control liver, B: Fusogenic peptide treated liver, C: Control lungs D; Fusogenic peptide treated lungs.

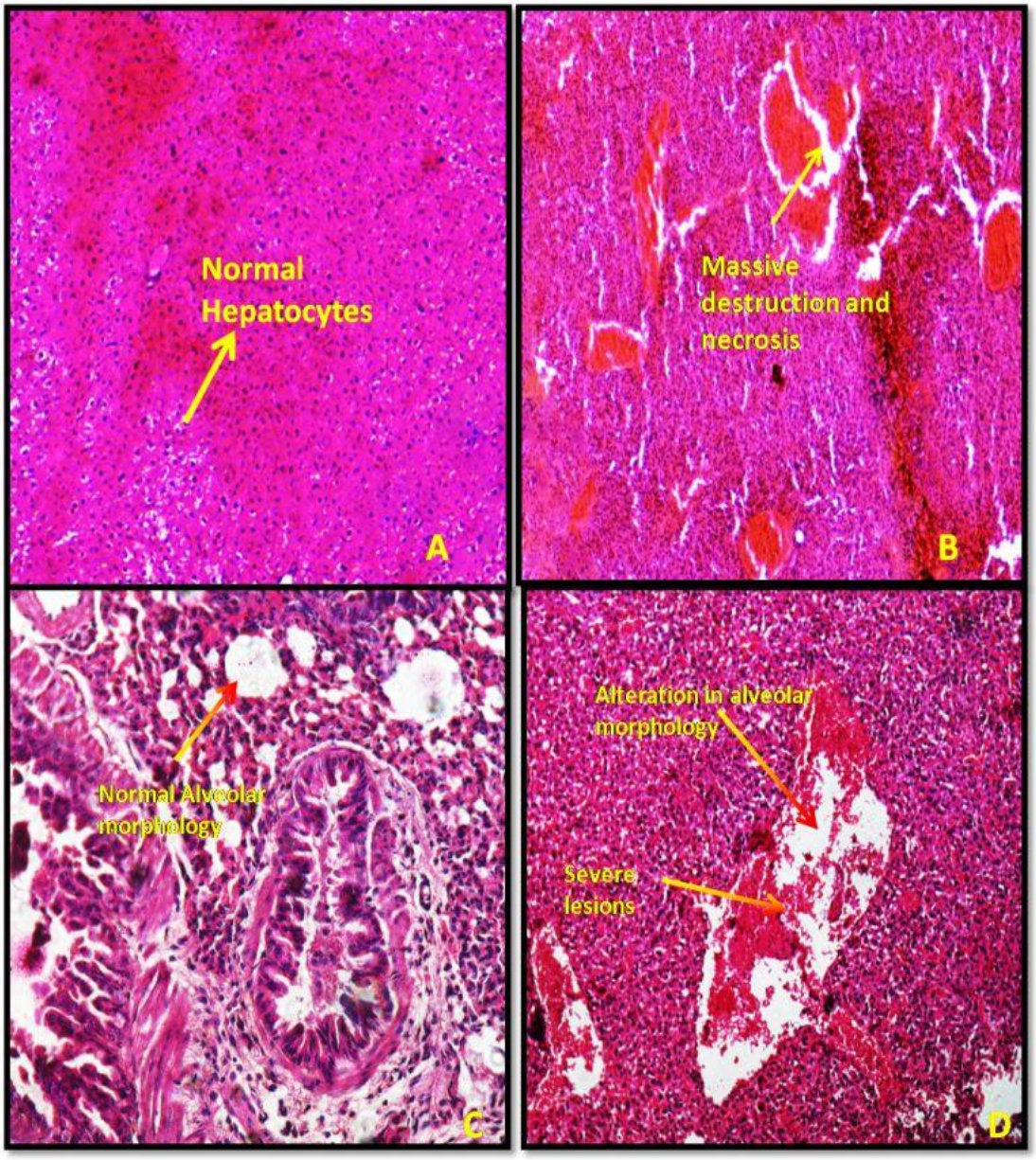
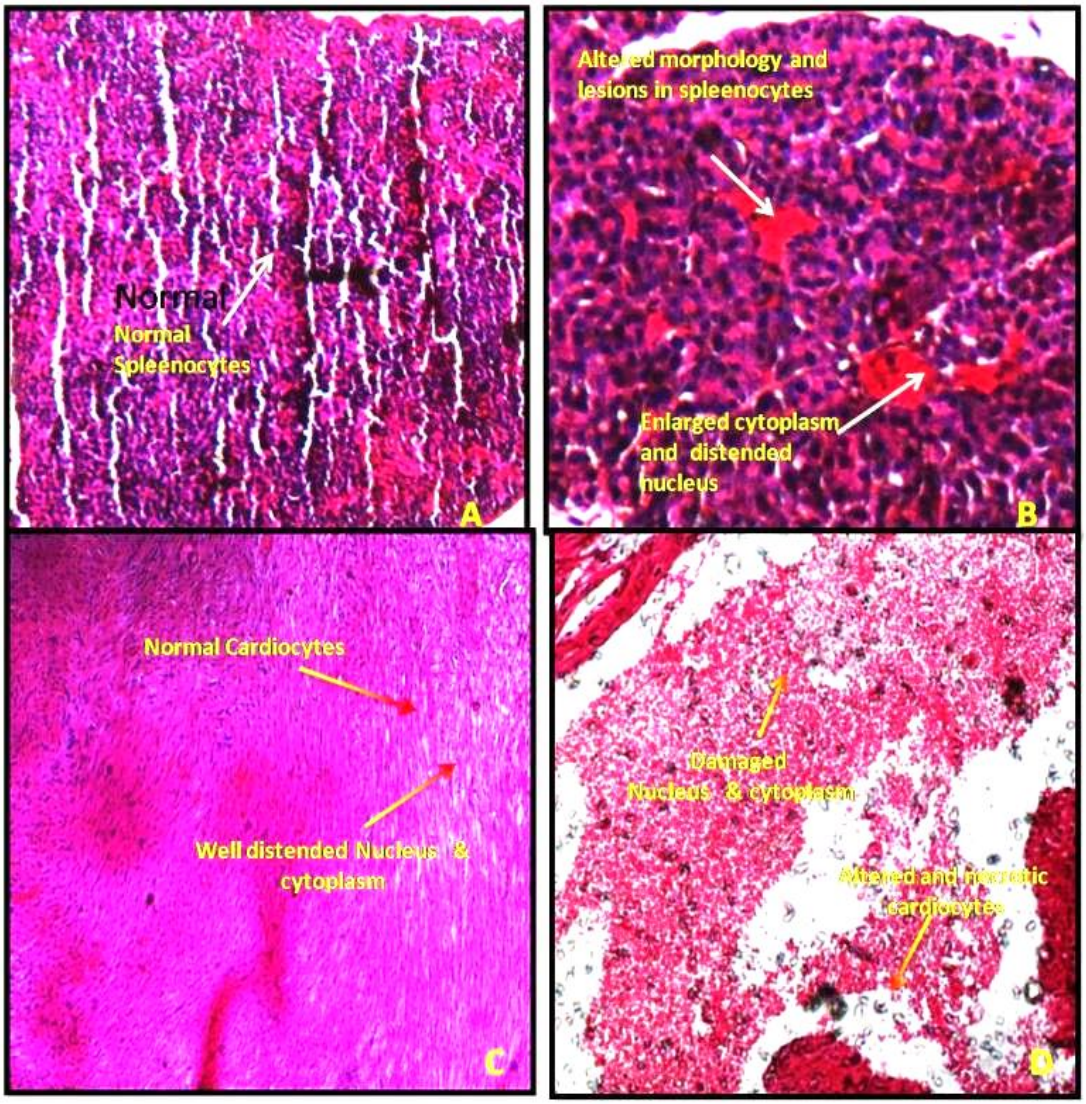


Figure 6.28: Histopathological study of Fusogenic peptide (from flavivirus) in swiss albino mice

A: Control Spleen, B: Fusogenic peptide treated Spleen,
C: Control Heart D: Fusogenic peptide treated Heart

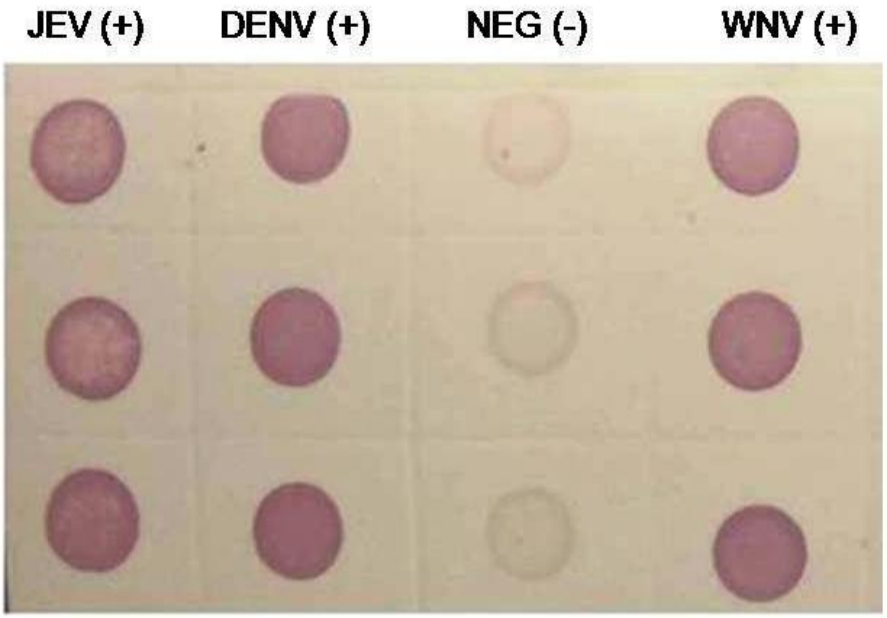


Similarly, the fusion peptide treated spleen showed that the alteration and lesions in the spleen of the mice displayed in the figure 6.28D. The control spleen showed the normal architecture and no such damage was seen in the control spleen (Figure 6.28C). The spleen and heart were abruptly damaged the spleenocytes were damaged well in the spleen and the cardio cells and the myocytes in the heart was damaged. Fusion peptide treated heart showed there was a massive damage in the cytoplasmic cells.

The fusion peptide treated lungs showed the disorted alveolar morphology (Figure 28B) and there was an observation of damaged cells due to toxicity when compared to the control lungs in the control Swiss Albino mice. The pathological observations in the figure 6.28B & 6.28D confirms that the fusion peptide is highly toxic to the animals. Further, the conserved sequences of all three flavivirus exhibited high toxic potential when challenged in Swiss Albino mice model.

Finally, mice hyper immune sera was developed for evaluating the antigen (peptide) specific response in the treated mice.

Figure 6.29: Dot ELISA. 1.JEV; 2. DENV; 3.NEG and 4.WNV



Serum showed high titre of antibodies when collected from different time intervals. There was no color development in the pre immune sera and also in the adjuvant sera. The sera were specific as they interacted only with viruses and not with other antigens such as Chikungunya, HSV, Influenza and Measles presented in the figure 6.29. The Dot ELISA was developed and presented in the figure 6.29.

DISCUSSION

Flavivirus causing Acute Encephalitis Syndrome (AES) is a major public health problem in India which mainly affects the children and young adults. The first major outbreak was in West Bengal in 1973 involving 700 cases and over 300 deaths. The AES is primarily caused by falvivirus. The group within the flavivirus genus with the largest number of viral species transmitted by mosquito vectors. These species include the Dengue virus (DENV), Japanese Encephalitis Virus (JEV), Myovascular Encephalitis Virus (MVEV), St. Louis Encephalitis Virus (SLEV), West Nile Virus (WNV), Yaounde virus, Cacipacore virus, Koutango virus, and Usutu virus. Among the above viruses the JEV, DENV and WNV were predominantly spread by the mosquito vector[271]. Dengue is caused by 4 genetically related but antigenically different viruses, and although it is uncertain where DENV evolved, maintenance of all 4 serotypes in enzootic cycles in Africa suggests that a progenitor virus most likely originated in Africa[130]. *Aedesaegypti* mosquitoes, the principal DENV vector, originated in Africa and spread to other countries in Africa and other tropical countries in the 17th and 18th centuries[271,272].

Several other *Aedes* species mosquitoes including *Ae.albopictus, Ae. africanus, and Ae. luteocephalus* are found in Africa and are potential DENV vectors[130] the 'domesticated' form is rarely found more than hundred from human habitation and feeds almost exclusively on human blood. West Nile virus is also an Arbovirus of the flavivirus family and is similar to other arthropod-borne flaviviruses that cause encephalitis, such as St Louis encephalitis, Japanese Polynesia virus, and Murray Valley virus.

The JE and WNV is transmitted by female mosquitoes, usually the *Culex* species and birds serve as amplifying host[272]. Patients in whom WNV is suspected can have either serum or cerebrospinal fluid examined for immunoglobulin M antibodies to WNV. The mortality rate for WNV in hospitalized patients is estimated to be between 10% and 15%; patients with both encephalitis and weakness have a mortality rate of approximately 30%[273]. Individuals recently infected with or

vaccinated against similar viral illnesses such as yellow fever, Japanese Encephalitis or Dengue can have false positive reactions for WNV[274] which are included in AES cases.

Dengue had traditionally been known to be a disease of high population dense tropical urban areas[275,276]. The proportion of rural and urban patients among those testing antibody positive more or less paralleled the rural-urban ratio of enrolled patients indicating that Dengue transmission is occurring equally in rural and urban areas. Over the last One-two decades there are increasing reports of Dengue cases and outbreaks from rural areas in Southern and Western India[273,277-282]. A single study from North India reports on outbreak of DF in rural Haryana[283]. These outbreaks were investigated in a small number of patients with Dengue antibody estimations. Therefore, the present study is focused to investigate the epidemiology and molecular characterization of JEV, DENV and WNV. These viruses vary widely in their ability and characteristics to infect human and animal species. Serological studies identified the Japanese Encephalitis (JE) virus as one of the key virus to cause AES mortality. In the year 1978 and 2007, over 100,000 cases of AES with a case fatality rate (CFR) of almost 33% were reported from 13 different states. It was widely presumed that JE was the predominant etiological agent in these outbreaks[284].

The year wise AES cases were studied from 2011 to 14, which totally included 2559 susceptible AES cases were investigated for the screening of JE, DENV and WNV viruses. Amid the suspected cases 127 cases were confirmed positive cases for JE IgM antibodies, 60 cases were positive for Dengue IgM antibodies and 13 cases were positive for WNV IgM antibodies. Further, the epidemiological studies indicate that there is an increase in the susceptible cases from 2011 to 2014. The number of positive cases were highly significant in the year 2013 followed by 2014.

The age wise distribution of the AES case study demonstrated that the pediatric age group ranged between 0-12 years revealed higher positivity than the rest of the other age groups. Among the positivity of AES cases, the JEV, DENV

and WNV viruses indicated high positivity in the year 2012. Similarly the confirmed positive cases of serum samples were further investigated in CSF samples. Higher percentage positivity were revealed in both JEV and DENV in the year 2012. Approximately 2 billion people live in countries where JE presents a significant risk to humans, particularly in China and India, with at least 700 million potentially susceptible children[285]. In worldwide thirty-five thousand to 50,000 cases of AES - JEV were identified each year with upto 10,000 deaths.

The JEV is the most common arbovirus causing human disease and the most frequent cause of childhood encephalitis in the endemic regions[285,286]. Encephalitis can only be said to have occurred when a histological diagnosis is available[279,284]. Several studies, however, had diagnosed encephalitis on indirect evidence, including absence of other explanations for encephalopathy, isolation of virus in CSF or its serological evidence, CSF pleocytosis, or focal neurological signs[275].

The district wise distribution revealed that Dengue occurred uniformly in the catchment area without areas of predilection. In the present study, the incidence of JEV, DENV and WNV causing AES in the various districts of Tamil Nadu was investigated during the period of 2011 to 14. The study revealed that the widespread JEV and DENV infection by vector transmission highly occurred in areas such as Chennai, Thiruvallur and Kanchipuram districts. In the same way the WNV were highly reported in the areas of Thiruvannamalai, Chennai and Thiruvallur districts.

All the reported areas were statistically significant and it was confirmed by the positive cases in subsequent areas. Season wise distribution were investigated during the study period between 2011 to 2014. The study revealed that the JE virus significantly reported as higher positivity in all the seasons except premonsoon. The Dengue virus explored highly in the North East and South West monsoon of 2011 to 2014 whereas West Nile virus explored only in the South West monsoon and not in the rest of the seasons.

Globally, there is seasonality variation reported in other countries. For example, the Eastern China, where JE cases were reported in summer season[277]. Higher number of cases may be due to creation of many breeding sources for Culex

mosquito species in paddy fields and might be responsible for the increased risk of JE cases[277,278]. Sero epidemiological surveys were also conducted in winter season (that is, November, 2005 to January, 2006) where 7.6% of JE cases were reported. JE cases during winter season may be due to second phase cultivation period of paddy when there were sufficient numbers of mosquito breeding places. Similar kinds of data were also reported on JE cases during winter season[278].

Based on these study reports, JE tends to be endemic and cases occur sporadically throughout the year with a peak after the start of the rainy season. Hence, it is assumed that climate variability has a direct influence on JE cases[279]. Temperature (22 to 34°C) and relative humidity (42.7 to 69.6%) were ambient conditions to facilitate the higher mosquito density as well as JE virus replication which in turn would be responsible for the occurrence of JE cases[287].

Month wise data of AES cases revealed that the higher monthly infective rate values were reported in the September to November and extended up to December and was least during summer season of year. This suggests that variations in infection during these different seasons are mainly responsible for transmission of vectors in humans. Thus, temporal changes might have greatly impacted the efficiency of arboviral transmission in nature, which would have significant epidemiological importance. Further it is noticed that minimum and maximum temperature were found to be positively correlated with number of AES cases in rainfall and relative humidity. It is also noticed that the average rainfall during the study period was 70.5mm and relative humidity was between (45.6 to 70.1%). Even though, with this low rainfall and low relative humidity, occurrence of many JE cases were noticed, and this may be due to the availability of sufficient number of breeding places for mosquitoes and suitable temperature for development and transmission of JEV and DENV virus.

More number of patients belonged to the age group of 11 to 20 years followed by 21 to 30 years which was consistent with studies conducted in different parts of India[288,289]. In a study conducted in Delhi 21 to 30 years age group was most commonly affected and another study conducted in Kanpur, showed 0 to 15 years

age group to be commonly affected[290,291]. In this retrospective study, DF was found to be the most common presentation which was similar to a study conducted in Delhi[292]. It is an established fact that complications like DHF and DSS occur mainly in cases with secondary infections due to antibody mediated immune enhancement, cross reactive T–cell response with activation of TH-2 lineage cell and stimulation of soluble factors[293]. In the present study the age wise distribution of the AES cases were comparatively high in the pediatrics and the young age group of 13-25. The other age groups were not significant to the positivity of AES investigation.

This longitudinal study revealed a steady increase of JE cases from 2011 to 2014, indicating a possible public health threat in the near future. The incidence of JEV and DENV infection was relatively high during South West and North East monsoon and it strongly depends on rainfall, humidity and temperature as well as the paddy cultivation. Effects of climate change on rainfall, temperature and other climatic variables may increase the vector populations and risk of JEV infection, especially in temperate regions like India. Apart from these factors, availability of high mosquito abundance, virus reservoirs, virus infected mosquitoes, development of resistance to effective insecticides, rapid globalization, population explosion and global climate change have also influenced the endemicity of this disease[281]. As of now, no disease surveillance studies have been carried out in this region, and this study is to make an attempt to understand the disease scenario and vector dynamics in relation with weather variables.

Further the maximum number of Dengue cases were reported in the month of September which indicated an active viral transmission during monsoon and post-monsoon period as reported earlier[282,284]. A higher occurrence of Dengue infection was noted among females which is similar to the present study conducted in Chennai[294]. However, this was discordant with other studies where a male predominance was noticed. Similarly in the present study the gender wise analysis indicated that the male population of the AES cases was highly susceptible for all the flaviviruses. The overall statistical analysis of JEV, DENV and WNV revealed the higher numbers of positive cases were occurred in the male population when compared to the female population.

Further the molecular characterization of JEV, DENV and WNV were genetically sequenced with the non structural protein gene 5 and polyprotein gene. The PCR amplicons of the non structural protein gene 5 and polyprotein genes were sequenced and detected for the genotypic characterization. The study indicates that the JEV was characterized by non structural region of 5 with 241bp and notably it was developed as markers for diagnosis. Similarly the DENV were characterized by two genotypes which include D3 and D4. The D3 genotype of DENV was characterized by 288bp and D4 genotype of DENV was characterized by 260bp. The WNV were characterized by the 396bp polyprotein gene.

Similarly recent reports identified the presence of WNV infection in Southern India by real-time RT-PCR and RT- LAMP assays and found neuroretinitis and retinitis to be the most common ocular findings. The study inferred that the majority of patients had a good prognosis. Real-time RT-PCR and RT-LAMP assays can be used as diagnostic tools, and the RT-LAMP assay is more cost-effective with the same effectiveness as RT-PCR and real-time RT-PCR. Real-time RT-PCR assays using either probes with Taqman technology or SYBR Green have been developed for the detection of WNV infection[295,296]. Recently, other PCR-based methods such as the RT-LAMP assay have been developed by targeting the envelope gene of WNV. The RTLAMP assay is a novel method of gene amplification that amplifies nucleic acid with high specificity, efficiency and rapidity, under isothermal conditions, with a set of six specially designed primers that recognize eight distinct sequences of the target[297].

Haemagglutination (HA) and Haemagglutination Inhibition assays (HI) have been widely used for the detection of arthropod-borne viruses and the antibodies developed to these viruses, respectively[298]. These assays exploit the ability of the envelope glycoprotein to bind and agglutinate avian erythrocytes so that they form a visible lattice in a U-bottom microtitre plate. In the HI assay, antibodies from infected individuals prevent the agglutination of the erythrocytes, which subsequently form a pellet.

While the HAI assay was used extensively in the past for virus serology, this technique has now been largely superseded, in favour of assays with better sensitivity and specificity, but is still used in some instances for surveillance. The advantages of HAI assays were providing avian red blood cells are available, the assays can be performed with minimal training and equipment and the antigen used could be inactivated by a simple extraction process. However, as for PRNT, there is a requirement for the simultaneous assessment for viruses endemic for the area and multiple different pH buffers are required for each different antigen.

In the present study the flavivirus isolated samples were subjected for Haemagglutination in different pH. The study reveals that the optimum pH for the Haemagglutination of the JEV, DENV and WNV. JEV was well agglutinated at pH 6.2. Similarly the Dengue and West Nile viruses were agglutinated well at pH 6.4. Further the study indicated that the HA viral titre of JE viruses was optimally observed as 1:128, Dengue viruses demonstrated the optimum titre of 1:256 and West Nile virus as 1:32.

The Immunofluorescence assay (IFA) can be used to differentiate the IgM and IgG responses to viral infection. It involved incubation of patient serum with glass slides, upon which were fixed virus infected cells. The benefit of this assay is that prefixed slides can be stored at 4°C and a BSL-3 facility is not required to perform the assay and results can be obtained quickly, particularly due to the commercialization of IFA kits (e.g., Focus Diagnostics Arbovirus IFA).

However, cross-reactivity of immune antibodies with closely related viruses can impair the accuracy of the diagnosis and there is a requirement for a fluorescent microscope to evaluate the results. In the study JEV, DENV and WNV isolates were confirmed by IFA and illustrated as apple green colour under immunofluorescence microscope. The negative of all the viruses were cherry red colour due to the non adherent binding of specific IgM antibody fluorescent tag. Further in the study it was standardized as the dilution of 1:10 stains good fluorescence intensity with less noise in the background. This observed dilution was further applied to the studies with the viruses isolated from clinical samples.

Nucleic acid-based assays offer rapid and specific detection and serotyping of viruses are gradually replacing serological and culture techniques. These methods include nested RT-PCR real-time RT-PCR, loop-mediated isothermal amplification, nucleic acid sequence-based amplification and Taqman assays[299].

In the present study the molecular phylogeny, of JEV isolates i.e. [Genbank :KJ947880] were very closer to the clade of Indian sequences gi|496909/India/2005/envelope, gi|239633849/India/2009/envelope and gi| 289466091/ India/2011/envelope. This showed that closer clustering, even though all of 2 isolates were from a geographically distant location or different districts of the state of Maharashtra, Kerala and Assam. This indicated similarities among the isolates circulating in the 2 consecutive years 2009 and 2011.

Moreover, based on the present study, stated that there had been simultaneous circulation of JEV strains in India. Similarly the Dengue strains were highly close to the clade of Taiwan (gi/25992032/Taiwan/2006/Polyprotein) (gi/25992032/Taiwan/2006/Polyprotein), Peru (gi/19071812 Peru/2002/capsid) and Srilanka (gi 164654854 Srilanka/2015/Anchored). The above clade were highly close to the KIPM KJ947880 sequences. In the West Nile virus the phylogeny indicated that WNV 1 and 2 were highly homologous with the clade of Madagascar (gi|58618099/Madagascar/2006/polyprotein) and USA (gi|27735298/ USA/2012/unnamed). Further the strain was also homologous (94%) with Israel (gi|290246783/Israel/2011/polyprotein) and USA gi|158516889/USA/2015/ unnamed).

SUMMARY

Acute encephalitis syndrome (AES) is a clinical condition caused by infection with viruses such as Japanese Encephalitis virus (JEV), Dengue viruses and West Nile viruses or other infectious and noninfectious causes. Acute Encephalitis Syndrome (AES) causes a great public health problem in India, occurring both in epidemics and sporadically. Globally, the magnitude of problem has been estimated to be around 50,000 cases and 10,000 deaths annually[300].

A confirmed etiology is generally not required for the clinical management of AES. Thus, surveillance of the above viral infections in India has focused on identifying AES cases rather than JEV, DENV and WNV cases. AES in India, especially Northern India, as per definition of WHO[301,302] encompasses several other illnesses; it has malaria, enteric encephalopathy, tubercular meningitis, Dengue with neurological manifestations, scrub typhus, bacterial meningitis, etc. It surely is a mixed pot.

Japanese Encephalitis, an arboviral infection, is a serious public health problem in the Asian region. Globally, it affects 67,900 people per year, of which about a third are likely to die. The change in climate, ecological imbalance, and population growth together with related demands for change in agriculture and animal rearing practices have recently intensified its threat along with that of other viral encephalitis. However, many nations still struggle for disease burden data on acute encephalitis that can be used to plan prevention and control strategies. Man is the accidental host and dead end for the transmission of the disease[303,304].

In Tamil Nadu, the first clinical case of JE was observed in 1955 at Vellore in India[305]. The first major Outbreak of JE occurred in 1973 in Bankura & Burdwan districts of West Bengal. In1976, wide spread outbreaks were reported from Andhra Pradesh, Assam, Karnataka, Tamil Nadu, Uttar Pradesh and West Bengal. The sources of virus may be different causing almost similar symptoms. Hence, all JE cases are being reported under Acute Encephalitis Syndrome (AES) after the outbreak of JE in Gorakhpur and Basti divisions in Eastern Uttar Pradesh during

2005 [302]. It is a disease of major public health importance because of its epidemic potential and high case fatality rate. The highly affected states include Andhra Pradesh, Assam, Bihar, Goa, Karnataka, Manipur, Tamil Nadu, Uttar Pradesh and West Bengal. Outbreaks of JE, Dengue and WNV usually coincide with monsoons and post- monsoon period when the vector density is high. Early management of the disease is essential, because there is no specific treatment. High vaccine coverage along with active surveillance is essential.

The ultimate objective is to prevent the disease occurrence by early diagnosis, implementation of effective control measures, high vaccine coverage with strong and active surveillance system[306]. Therefore in the present study the sero surveillance of AES including viruses such as Japanese Encephalitis, Dengue and West Nile viruses were investigated in to 14 districts of TamilNadu viz., Chennai, Thiruvallur, Kanchipuram, Thiruvannamalai, Villupuram, Cuddalore, Thanjavur, Ariyalur, Perambalur, Vellore, Trichy, Madurai, Thirunelveli and Erode.

Among the districts, the maximum positive AES cases of JE and Dengue were reported in Chennai, Thiruvallur and Kanchipuram district of Tamil Nadu. The higher positive AES cases of West Nile virus were explored in Thiruvannamalai, Chennai and Thiruvallur. The age wise distribution of AES cases were analyzed and divided like 0-12, 13-18, 19-35, 36-55 and above 55. The prevalence in different age groups were statistically analyzed and represented in the figure by standard error mean. Among the age groups, the positivity was predominantly seen in the pediatric age group in all the years. Few positive cases were observed in young adults.

In the years 2013 and 2014, the high positivity was observed in adults and elder age groups. The total suspected cases were statistically analyzed using linear regression in different periods from 2011 to 2014. The linear regression analysis indicates that there is an increase in sample size and the total number of suspected cases was linearly raised from 2011 to 2014. In comparison of the three viruses in AES cases the Dengue occupies higher number of samples. The sample size of all the viruses in AES in the study period 2011-14 was in the order of JEV > Dengue>West Nile viruses. But the regression scatter plot in reveals that all the

three flaviviruses were increase in the susceptible cases. The multiple comparison analysis of the study period infers the higher number of positivity was observed in 2013 followed by 2014.The Post hoc Dunnet statistical analysis inferred that there was significant difference was found within the groups and there was no significant difference between the groups. The one way ANOVA analysis of different age groups were analyzed in different periods of the year. The mean difference was significant in all the age groups with degrees of freedom 0.05 level.

The season wise distribution of cases was statistically analyzed for their epidemiological characterization of the AES in the study period. The Japanese Encephalitis virus expresses predominantly highest positive cases in all the seasons except premonsoon. The Dengue viruses have highest positive cases in the South West and North East monsoon seasons. Less number of susceptible cases occurs during the winter and premonsoon seasons. The Positive cases of West Nile virus is highly occurring in the south west monsoon than the other seasons. The age wise and gender wise analysis were done in the study period. It was observed that the male cases were high positive and susceptible than the female cases. Especially the age group of pediatrics (0-12) and young age (12-25) group indicated high positivity than the rest of the other age groups.

The year wise AES Positive cases were studied from 2011-14, totally 2559 susceptible AES cases were investigated for the screening of serum ELISA in JE, DENV and WNV viruses. Amid the susceptible cases 127 cases were confirmed positive cases for JE IGM antibodies, 60 cases were positive for serum IGM antibodies and 13 cases were positive for WNV IGM antibodies.

Further the epidemiological studies indicate that there is an increase in the susceptible cases from 2011-14. But the number of positive cases was highly significant in the year 2012. Because less number of cases were screened in 2012, but high number of positives were confirmed. Similarly CSF cases were screened totally in 1792 samples. High numbers of positivity were revealed in both JEV and DENV than WNV. Further the viruses were characterized molecularly and genotypically for developing new markers for diagnosis. The positive samples were

cultured in the Vero cell lines. Then it the specificity and sensitivity of the cell passages were characterized.

The isolated JE virus were more sensitive in the early passages of 2 and 4, rest of the passages were less sensitive and specificity. Similarly, the Dengue virus was more attuned in the late 6th passage. But the West Nile virus was highly compatible in the 5th passage of Vero cell lines.

The Virus isolated samples were then preceded for Haemagglutination in different pH (6, 6.2 and 6.4). The optimum pH of the JE virus was 6.2, Dengue and West Nile viruses were agglutinated well at the optimum pH of 6.4. The viral titre of JE viruses was observed as 1:128. Dengue viruses revealed the optimum titre of 1:256 and west Nile virus 1:32. For the rapid diagnosis, Immunofluroscence assay was attempted in the JEV, DENV and WNV.

Further in the study it was standardized as the dilution of 1:10 stains good fluorescence intensity with less noise in the background. This observed dilution was further applied to the studies with the viruses isolated from clinical samples. Further in the present study, the molecular phylogeny of JEV isolates i.e. [Genbank :KJ947880] were found to be closer to the clade of Indian sequences gi|496909/India/2005/envelope, gi|239633849/India/2009/envelope and gi| 289466091/India/2011/envelope. This indicates that closer clustering, even though all of 2 isolates were from a geographically distant location or different districts of the state of Maharashtra, Kerala and Assam. This demonstrated that similarities among the isolates circulating in the 2 consecutive years 2009 and 2011.

Moreover, based on the present study, stated that there had been simultaneous circulation of JEV strains in India. Similarly the Dengue strains were highly close to the clade of Taiwan (gi/25992032/Taiwan/2006/Polyprotein) (gi/25992032/Taiwan/2006/Polyprotein), Peru (gi/19071812 Peru/2002/capsid) and Sri Lanka (gi 164654854 srilanka/2015/Anchored). The above clades were highly close to the KIPM KJ947880 sequences.

In the west Nile virus the phylogeny indicated that WNV 1 and 2 were highly homologous with the clade of Madagascar (gi|58618099/ Madagascar/ 2006/polyprotein) and USA (gi|27735298/ USA/2012/unnamed). Further the strain was also homologous (94%) with Israel (gi|290246783/Israel/2011/polyprotein) and USA gi|158516889/ USA/2015/unnamed). Finally, this indicates that the same type of West Nile strains was circulated in India. Further the strains were exposed due to the patients recently migrated in the homologues countries and spread in Southern part of India.

CONCLUSION

- The role of three flaviviruses namely, JEV, DENV and WNV viruses as causative agent of Acute encephalitis syndrome during the period 2011-2014, their epidemiology and molecular characterization were analyzed in depth.

- Among the three flaviviruses screened, JEV was found to be most prevalent, followed by DENV and then WNV.

- The pediatric age group (0-12 yrs) showed a high positivity for flaviviruses followed by the young and adolescent age group of 13-25 years.

- Out of the 14 districts screened the district Chennai, Thiruvallur, Thiruvannamalai and Kanchipuram districts had high prevalence of AES viruses such as JEV/DENV/WNV during the study period of 2011-2014. JEV infection was highest in Chennai, Thiruvallur and Kanchipuram districts. DENV infections were found to be high in Thiruvannamalai districts. WNV causing AES in Tamil Nadu was found to be high in Chennai district followed by Kancheepuram. .

- Season wise distribution of the study demonstrates that the JEV positivity was widespread in all the seasons more during the monsoon and post monsoon season, but low degree was positivity was seen during summer months also showing residual activity of mosquitoes. The Dengue virus positivity during the months of September, October and elevated in November. In the districts along the Western ghats, like Theni, Kanyakumari, Thirunelveli DENV positivity started early, from end of June onwards raising gradually and peaking in November and December months.

- These districts receive rains early due to the South West monsoon and their positivity increases with rains and post monsoon months have high positivity due to water stagnation and storage. In the Northern district of Tamil Nadu the increase in positive cases, mainly due to North East monsoon which

starts from September end onwards. All this indicates public health activity should increase before the onset of monsoon with round the year public health department educate and create awareness on these infections.

- WNV infections were prevalent in the Northern districts and the early start of monsoon resulting in increase in their positivity.

- Month wise data of AES cases revealed that the higher MIR values were reported in the September to November and extended up to December and was least during only in summer season.

- During the study period, there is an increase in the susceptible cases from 2011-14. But there was a marked raise in the positivity of flavivirus infections in 2013 and 2014, possibly better health care facilities referring more cases for testing.

- Sera diagnosis indicated the prevalence of JE, showed positivity in the pediatric age group predominantly but during the course of the study there was an increase of JEV infections among the adult population.

- The molecular characterization of JEV, DENV and WNV was characterized by RT –PCR and nucleotide sequencing. On sequence analysis of JEV, the strains were found to be similar to the isolates similar to different districts of the state of Maharashtra, Kerala and Assam strains.

- WNV were very close to the clades of Madagascar and USA. strains on sequence analysis. Among DENV Subtype were predominant homologues to Taiwan, Peru and Srilanka. The molecular phylogeny of JEV isolates were homologues to Maharashtra, Kerala and Assam circulating in the 2 consecutive years 2009 and 2011 indicating that no new JEV strains were in circulation in Tamil Nadu.

- But the Dengue and West Nile isolates were homologues to Taiwan, Peru, Srilanka and USA and Madagascar. There is no mutant strain found in the isolates.

- The IFA assays were standardized for the simultaneous screening of JEV, DENV and WNV among AES cases. This provides a rapid diagnosis of flaviviruses especially in tertiary care setting.

- Standardization of RT-PCR, Peptide ELISA and Dot ELISA were developing new insight in marker development for JEV, DENV and WNV Diagnosis.

RECOMMENDATION

Vector control measures should be implemented for effective management and prevention of viral transmission such as Japanese Encephalitis, Dengue and West Nile viruses. Reducing contact between humans and potentially infected mosquitoes is the only way to reduce infection rates, morbidity and mortality due to viral infection. Personal protective measures that should reduce the risk of infection by avoiding outdoor activity in areas with potentially infected mosquitoes, using mosquito repellents, eliminating conditions that support mosquito breeding (eg, eliminate standing water, clean rain gutters); maintaining window and door screens, wearing long-sleeved shirts and long pants in outdoors.

Vaccination programme should be advised to public and create awareness among the public on the severity of viral diseases such as JE, Dengue and West Nile viruses. Available Vaccinations such as JE virus should be reach people living in susceptible areas for effective management and prevention. Other viruses such as Dengue and WNV infected patients should be quarantined if affected and necessary steps should be taken for treatment and prevention. Department of public health and medical education should take necessary steps for controlling vectors and educate the people frequently through medias in the highly susceptible places. Finally government should support and strengthen the laboratories for surveillance and incidence of flaviviruses (JE, Dengue and WNV) in all the reported areas.

BIBLIOGRAPHY

1. Karabatsos, N., 1985. International catalogue of arboviruses, 3rd. American Society of Tropical Medicine and Hygiene, San Antonio Tex.

2. Murphy, F.A., Fauquet, C.M., Bisho, D.H.L., Ghabtial S.A., et al. 1995. Virus taxonomy: classification and nomenclature of virus, 415-421. Spring-Verlag, New York, N.Y.

3. Halstead, S.B., Jacobson J. 2003. Japanese encephalitis. Adv Virus Res 61:103.

4. Monath, T.P. 1994. Yellow fever and Dengue the interactions of virus, vector and host in the re-emergence of epidemic disease. SeminVirol 5:133-145.

5. Le Guenno, B., Bougermouh, A., Azzam, T., Bouakaz, R. 1996. West Nile: a deadly virus? Lancet 348-1315.

6. Tomori, O. 2004. Yellow fever: The recurring plague, Crit Rev Clin Lab Sci. 41:391-427.

7. Mc Caw, W.D., Walter Reed: A Memoir. Yellow fever: A Compilation of various publications. Results of the work of Maj. Walter Reed, Medical corps, United States Army, and the Yellow Fever commission. 1911:8-12.

8. North Dakota Department of Health: Department of preparedness & Response. Flavivirus fact sheet. Accessed March 3 2011. 2005. North Dakota Department of Health. http://www.ndhealth.gov/EPR/public/viral/FlavivirusFact.htm.

9. Tomori, O., 2004. Yellow fever: The recurring plague. Crit.Rev.Clin Lab Sci. 41:391-427.

10. Mackenzie, J.S., Gubler, D.J., Petersen, L.R. 2004. Emerging Flaviviruses: The spread and resurgence of JE, West Nile and Dengue viruses. Nat. Med. 10: S98-109.

11. Weaver, S.C., Barrett, A.D. 2004. Transmission cycles, host range, evolution and emergence of arboviral disease. Nat Rev Microbiol. 2:789-801.

12. Gubler, D.J. 2011 Emerging vector Borne flaviviral diseases: are vaccines the solution? Expert Rev Vaccines. 10(5):563-5.

13. Duane, J.G., Paul Reiter., Kristie, L Ebi., WandyYap., Roger Nasci, et al. 2001. Climate variability and change in the United states: potential impacts on vector and rodent-borne diseases. Enviro. Health Perspectives. 109(2):223-233.

14. Van den Hurka, F., Ritchie S.A., Mackenzie J.S. 2009. Ecology and geographical expansion of Japanese encephalitis virus. Annu Rev Entomol. 54:17-35.

15. Erlanger T.E., Weiss S., Keiser J., Utzinger, J., et al. 2009. Past, present and future of Japanese encephalitis. Emer Infect Dis. 15:1-7.

16. Guzman, M.G., kouri, G. 2003. Dengue and Dengue hemorrhagic fever in the Americas: lessons and challenges. J Clin Virol 27:1-13.

17. Priya, S. 2003. West Nile: Epidemiology, clinical presentation, diagnosis and prevention. Mayo Clin Proc. 78:1137-1144.

18. Ramakrishnan, S.P., Gelfand, H.M., Bose, P.N., Sehgal, P.N., et al. 1964. The epidemic of acute hemorrhagic fever Calcutta, Epidemiology inquiry Ind J Med Res. 52:633-650.

19. Kaul, S.M., Sharma, R.S., Sharma, S.N., Panigrahi, N., et al. 1998. The role of entomological surveillance J Com Dis 30:187-192.

20. World Health Organization:Technical information on Japanese encephalitis. Guidelines for surveillance and control SEA/CD/108/1988.

21. Sharma, R.C., Saxena, V.K., Mohan Bhardwaj., Sharma, R.S., et al. 1991. An outbreak of Japanese encephalitis in Haryana-1990. J Com Dis 23(2):168-169.

22. Murgue, B., Murris, Triki, H., 2001. West Nile in the Mediterranean basin:1950-2000. Ann NY Acad Sci. 951:117-126.

23. Jmor, F., Emsley, H.C.A., Fischer, M. et al. 2008. The incidence of acute encephalitis Syndrome in Western industrialized and tropical countries. Virol J 5:134.

24. Potharaju, N.R. 2012. Incidence rate of acute encephalitis syndrome without specific treatment in India and Nepal. Indian J Comm Med. 37:240-51.

25. Encephalitis toll touches 588 in Uttar Pradesh. rediff.com, rediff. news, December 19, 2012. Available at <http://www.rediff. com/news/report/encephalitis-toll-touches-588-in-uttar-pradesh/20121219.htm.

26. Saxena, V., Dhole, T.N. 2008. Preventive strategies for frequent outbreaks of Japanese encephalitis in northern India. J Biosci33:505–14.

27. Saxena, S.K., Mishra, N., Saxena, R., Singh, M., et al. 2009. Trend of Japanese encephalitis in north India: evidence from thirty-eight acute encephalitis cases and appraisal of niceties. J Infect Dev Ctries 30:517–30.

28. Chakraborty, S.K., Sarkar, J.K., Chakraborty, M.S., Mukherjee, K.K. et al., 1973. Outbreak of encephalitis in the coal mine area near Asansol—a preliminary note. Bull Calcutta Sch Trop Med 21:1–2.

29. Mukherjee, M.K., Sarkar, J.K., Chakraborty, S.K., Sen Gupta, S.K. et al. 1973. Outbreak of encephalitis due to Japanese encephalitis in Bankura—a preliminary report. Bull Calcutta Sch Trop Med 21:2–3.

30. Paul S., Gupta, N.P., Gupta, S.P. 1959. Enteric viruses from sporadic cases of aseptic meningitis encephalitis. J Indian Med Assoc 312–16.

31. Carey, D.E., Myers, R.M., Webb J.K., Reuben, R. 1969. Japanese encephalitis in South India.A summary of recent knowledge. J Indian Med Assoc 52:10–15.

32. Kabilan, L., Edwin, N., Balashankar, S., Meikandan, D. et al. 2000. Japanese encephalitis among paediatric patients with acute encephalitis syndrome in Tamil Nadu, India. Trans R Soc Trop Med Hyg 94:157–8.

33. Kumar, R., Mathur A., Kumar, A., Sethi, G.D. et al. 1990. Virological investigations of acute encephalopathy in India. Arch Dis Child 65:1227–30.

34. Potula, R., Badrinath, S., Srinivasan, S. 2003. Japanese encephalitis in and around Pondicherry, South India: A clinical appraisal and prognostic indicators for the outcome. J Trop Pediatr 49:48–53.

35. Kumar, R., Tripathi, P., Singh, S., Bannerji, G. 2006. Clinical features in children hospitalized during the 2005 epidemic of Japanese encephalitis in Uttar Pradesh, India. Clin Infect Dis 43:123–31.

36. Endy, T.P., Nisalak, A. 2002. Japanese encephalitis virus: Ecology and epidemiology. In: Mackenzie, J.S, Barrett, A.D.T., Deubel, V. (eds). Japanese encephalitis and West Nile viruses. Berlin, Heidelberg: Springer-Verlag 11–48.

37. Sinha, K.,Ghulam Nabi Azad. 2011. Prescribes measures to contain encephalitis outbreak in UP. Times of India 25 Oct.

38. Lidenbach, B.D., Rice, C.M. 2001. Flaviviridae: the viruses and their replication. In Fields Virology. 2:991-1041.

39. Dim mock, N.J., Easton A.J., K.N. et al. 2007. Introduction to modern virology.6:453-454.

40. Liao, C.L., Lin Y.L. 2002. Japanese encephalitis virus infection initiates endoplasmic reticulum stress and an unfold protein response. J Virol 76(9):4162-71.

41. Sarika, T., Rishi Kumar Singh, Ruchi T., Tapan, N.D. 2012. Japanese encephalitis: a review of the Indian perspective. Braz J Infect Dis 16(6):564-573.

42. Guzman, et al. 2010. A continuing global threat. Nature Reviews Microbiology 8:s7-s16.

43. Melanie, S.S., Michael, S.D. 2006. Pathogenesis of West Nile virus infection: a balance between virulence, innate and adaptive immunity and viral evasion. J Virology 80(19):9349-9360.

44. Mukhopadhyay, S., Kuhn, R.J., Rossmann, M.G. 2005. A structural perspective of the flavivirus life cycle. Nat Rev Microbiol 3(1):13-22.

45. Bradley, J.B., and Andrew, E.F. 2015. Insect-specific Flaviviruses: a systematic review of their discovery, host range, mode of transmission, super infection exclusion potential and genomic organization. Viruses 7(4):1927-1959.

46. Rosen, L., Lien, J.C., Shroyer, D.A., Baker, R.H., et al. 1989. Experimental vertical transmission of Japanese encephalitis virus by *Culex tritaeniorhynchus* and other mosquitoes. Is J Troop Med Hug (40):548–556.

47. Burke, D.S., Leake, C.J. 1988. Japanese encephalitis. In: Monath T.P. (Ed.), The Arboviruses: Epidemiology and Ecology, vol. III. CRC Press, Boca Raton F.L, 63-/92.

48. Scherer, W.F., Buescher, E.L., McClure, H.E. 1959. Ecologic studies of Japanese encephalitis virus in Japan. V:Avian factors. J Immunol(3):689–697.

49. Scherer, W.F., Buescher, E.L., Flemings, M.B., Noguchi, A. et al. 1959a. Ecologic studies of Japanese encephalitis virus in Japan. III. Mosquito factors. Zoo tropism and vertical flight of Culextritaeniorhynchus with observations on variations in collections from animal-baited traps in different habitats. Am J Trop MedHyg (8):665–677.

50. Gubler DJ. Epidemic dengue and dengue hemorrhagic fever: a global public health problem in the21st century. In: Scheld WM, Armstrong D, Hughes JM (Eds) ASM press, Washington, DC(1).

51. Vaughn, D.W., Green, S., Kalayanarooj, S. et al. 1997. Dengue in the early febrile phase:viremia and antibody responses. J Infect Dis 176:322.

52. Anderso,n J.F., Main, A.J., Andreadis, T.G., Wikel, S.K. et al. 2003. Transstadial transfer of West Nile virus by three species of Ixodid ticks (Acari:Ixodidae). J Med Entomology 40(4): 528–533.

53. Andreadis, T.G., Anderson, J.F., Vossbrinck, C.R., Main A.J. 2004. Epidemiology of West Nile virus in Connecticut: a five-year analysis of mosquito data 1999–2003. Vector Borne Zoonotic Diseases. 4(4):360–378.

54. Leary, D.R., Marfin, A.A., Montgomery, S.P, et al. 2004. The epidemic of West Nile virus in the United States, 2002: Vector Borne Zoonotic Disease 4: 61-70.

55. Risbud, A.R., Sharma V., Mohan Rao, C.V.R., Rodrigues, F.M. et al. 1991. Post-epidemic serological survey for JE virus antibodies in South Arco district (Tamil Nadu): Indian . J Med Res. 93: 1-5.

56. Sucharit, S., Surathin, K., Shrestha, S.R., et al. 1989. Vectors of Japanese encephalitis virus (JEV): species complexes of the vectors. J Trop Med Pub Health 20(4):611-21.

57. Tara K.H. et al. 2004. TKH Virology notes. Dengue fever. Https://denguemu.wordpress.com/vector-transmission.

58. Mattingly, P.F. 1957. Genetic aspects of the Aedesaegypti problem.I. Taxonomy and bionomics. Ann Trop Med Parasitol. 51:392-408.

59. Miller, B.R., Nasci, R.S., Godsey, M.S., Savage, H.M. et al. 2000. First field evidence for natural vertical transmission of WNV in Culexunivittatus complex mosquitoes from Rift valley province, Kenya. Is J Trod Med Hyg? 62:240.

60. Anonymous: Update; Surveillance for WNV in over wintering mosquitoes-New York, 2000: MMWR Morbwkly rep. 49: 178.

61. Nasci, R.S., Savage, H.M., White, D.J, Miller, J.R., et al. 2001. West Nile virus in overwintering Culex mosquitoes, New York city, 2000: Emerging infectious disease. 7: 742.

62. National Vector Borne Control Programme (NVBCP) Directorate of Health Services Ministry of Health and family welfare. Guidelines for surveillance of acute encephalitis syndrome (with special reference Japanese Encephalitis) Nov 2006.nvbdcp.gov.in/Doc/AES/20guidelines.

63. Solomon, T., andWinter, P.M., et al. 2004. Neurovirulence and host factors in flavivirusencephalitis evidence from clinical epidemiology. Arch Virol Suppl 18:161–170.

64. Gourie, Devi, M., Ravi, V., Shankar, S.K. 1995. Japanese encephalitis: an overview. In: Rose, C.F. (Ed.), Recent Advances in Tropical Neurology. Elsevier, Amsterdam,221–235.

65. Kalita, J., Misra, U.K. 1998. EEG in Japanese encephalitis: A clinic radiological correlation. Electoencephalogr Clin Neurophysiol 106:238–243.

66. Misra, U.K., Kalita, J. 2001. Seizures in Japanese encephalitis. J NeurolSci. 190:57–60.

67. Kalita, J., Misra, U.K., Pandey, S., Dhole, T.N. 2003. A comparison of clinical and radiological findings in adults and children with Japanese encephalitis. Arch Neurol 60(12):1760–1764.

68. Misra, U.K., Kalita, J. 1997. Movement disorders in Japanese encephalitis. J Neurol244:299–303.

69. Solomon, T., Kneen, R., Dung, N.M., Khanh, V.C. et al. 1998. Poliomyelitis-like illness due to Japanese encephalitis virus. Lancet 351:1094–1097.

70. Mohammed, S.S.J., Sarumathy, S and Ravichandiran ,V. 2014. A case report on Dengue encephalitis, seizure and pyrexia of unknown origin. World J Phar and Pharmaceutical sciences. 3(6):416-422.

71. Hayes, E.B. and Gubler, D.J. 2006. West Nile virus;epidemiology and clinical features of an emerging in the United States. Annu Rev Med 57:181-194.

72. Anninger, W.V., Lomeo, M.D., Dingle, J., et al. 2003. West Nile virus-associated optic neuritis and chorioretinitis. A J Ophthalmology 136:1183-1185.

73. Kuchtey, R.W., Kosmorsky, G.S., Martin, D., et al. 2003. Uveitis associated with West Nile virus infection. Arch Ophthalmology 121: 1648-1649.

74. Kulstad, E.B., and Wichter, M.D. 2003. West Nile encephalitis presenting as a stroke. Annual Emer Med 4:283.

75. Georges, A.J, Lesbordes, J.L., Georges-Courbet M.C., et al. 1987. Fatal hepatitis from West Nile virus. Ann Inst Pasteur Virology 138:237-244.

76. Burke, D.S., Lorsomrudee, W., Leake, C.J., Hoke, C.H., et al. 1985. Fatal outcome in Japanese Encephalitis. Am J Trop Med Hyg 34:1203-1210.

77. King, N.J., Getts, D.R., Getts, M.T., Rana, S. 2007. Immunopathology of flavivirus infections. Immune Cell Biol. 85:33-42.

78. Mathur, A., Kulshreshtha, R., Chaturvedi, U.C. 1989. Evidence for latency of Japanese encephalitis virus in T lymphocytes. J Gen Virol 70:461-465.

79. Rios, M., Zhang ,M.J., Griney, A., Srinivasan, K. et al. 2006. Monocytes macrophages are a potential target in human infection with West Nile virus through blood transfusion. Transfusion. 46: 659-667.

80. Hunsperger, E.A., and Roehrig, J.T. 2006. Temporal analyses of the neuropathogenesis of a West Nile virus infection in mice. J Neurovirol 12:129-139.

81. Gromeier, M., Bossert, B., Arti, M., Nomoto, A. et al. 1999. Dual stem loops within the poliovirus internal ribosomal entry site control neurovirulence. J Virology 73(2):958-964.

82. Feuer, R., Mena, I., Pagarigan, R.R., Harkins, S. et al. 2003. Coxsackievirus B3 and Neonatal CNS: the roles of stem cells, developing neurons and apoptosis infection, viral dissemination and disease. Am J Pathology 163(4):1379-1393.

83. Feuer, R., Pagarigan, R.R., Harking, S., Liu F., et al. 2005. Coxsackievirus targets proliferating neuronal progenitor cells in the neonatal CNS. J Neuroscience 25(9):2434-2444.

84. Kosugi, I., Shinmura, Y., Hawasaki, H. 2000. Cytomegalovirus infection of the CNS stem cells from mouse embryo: a model for development brain disorders induced by cytomegalovirus. Lab Investigation 80(9):1373-1383.

85. Das and Basu A., 2008. Inflammation a new candidate in modulating adult neurogenesis. J Neuroscience Res (86)6:1199-1208.

86. Sridhar, V.B., Matthew, J.K., Sherif, R.Z and James, J.S. 2014. Encephalitis caused by pathogens transmitted through organ transplants United States, 2002-2013. Emer Infect Dis 20(9):1433-1451.

87. Duane, J.G., 1998. Dengue and Dengue Hemorrhagic fever. Clin microbial Rev 11(3):480-496.

88. Hayes, E.B. and Gubler D.J. 1992. Dengue and dengue hemorrhagic fever. Pediatr infect Dis 11:311-317.

89. Sabin, S.B., 1952. Research on dengue world war II. Am J Trop Med Hyg 1:30-50.

90. Siler, J.F., Hall, M.W. 1926. Dengue, its history, epidemiology, mechanism of transmission, aetiology, clinical manifestations, immunity and prevention. Philipp J Sic 29:1-304.

91. Water man, S.H., Gubler, D.J. 1989. Dengue fever. ClinDermatol 7:117-122.

92. Chungue, E. 1997. Molecular epidemiology of dengue virus, In Saluzzo J.F., Dodet B. editors. Factors in the EmerArbo Dis 93-101.

93. Lanciotti, R.S., Lewis, J.L., Gubler, D.J., Trent, D.W. 1994. Molecular evolution and epidemiology of dengue-3 viruses. J Gen Virol 75:65-75.

94. Lewis, J.A., Chang, G.J., Lanciotti, R.S., Kinney, R.M. 1993. Phylogenetic relationship of dengue-2 virus. Virology 197:216-224.

95. Rico, H.R. 1990. Molecular evolution and distribution of dengue virus type 1 and 2 in nature. Virology 174:1479-493.

96. Rico, H.r., Harrison, L., Salas, R., Tovar, D. et al. 1997. Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 230:244-251.

97. Trent, D.W., Grant, J.A., Monath T.P., Manske, C.L. et al. 1989. Genetic variation and microevolution of dengue 2 virus in Southeast Asia. Virology 172:523-535.

98. Melanie, A.S and Michael, S., 2006. Pathogenesis of West Nile virus infection a balance between virulence. Innate and adaptive immunity and viral evasion. Virol 80(19):9349-9360.

99. Wang, T., Town, T., Alexopoulou, L., Anderson, J.F., et al. 2004. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat Med 10:1366-1373.

100. Diamond, M.S., Sitati E.M., Friend L.D., Higgs S. et al. 2003. A critical role for induced IgM in the protection against West Nile virus infection. J Exp med 198:1853-1862.

101. Garcia, T.D., Loiacono C.M and Kleiboeker. 2006. Replication of West Nile virus in equine peripheral blood mononuclear cells. Vet Immunol Immunopathol 110:229-244.

102. Kramer, HS., Rothenaigner, I., Wolff, H., Bell, J.E. et al. 2005. Cells of the central nervous system as targets and reservoirs of the human immunodeficiency virus. Virus Res 111:94-213.

103. Monath, T.P., Cropp, C.B and Harrison, A.K. 1983. Mode of entry of a neurotropic arbovirus into the central nervous system. Reinvestigation of an old controversy. Lab Inves 48:399-410.

104. Hunsperger, E.A and Roehrig, J.T. 2006. Temporal analyses of the neuropathogenesis of a West Nile infection in mice. J Neurovirol 12:129-139.

105. Johnson, R.T. 1998. Viral infections of the nervous system, 2nd ed. Lippincott Williams & Wilkins, Philadelphia, Pa.

106. Samuel, M.A and Diamond, M.S. 2005. Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival. J Virol 79:13350-13361.

107. World Health Organization. Outbreak encephalitis 2005: cases of Japanese encephalitis in Gorakhpur, Uttar Pradesh, India. 2005. Core Programme Clusters. Communicable Diseases and Disease Surveillance. 2005 Oct 21 [cited 2006 Jul 11]. Available from http://w3.whosea.org/en/Section1226/Section2073.asp.

108. Monath, T.P, Heinz, F.X. 1996. Flaviviridae. In: Fields B.N., Knipe, D.M., Howley, P.M, editors. Fields virology. 3rd ed. Philadelphia: Lippincott–Raven; 961–1034.

109. Solomon, T., Ni H., Beasley, D.W.C., Ekkelenkamp M. et al. 2003. Origin and evolution of Japanese encephalitis virus in Southeast Asia. J Virol77:3091–8.

110. Saxena , V., Dhole, T.N. 2008. Preventive strategies for frequent outbreaks of Japanese encephalitis in northern India. J Biosci 33:505–14.

111. Saxena, S.K., Mishra, N., Saxena, R., Singh, M. et al., 2009. Trend of Japanese encephalitis in north India: evidence from thirty-eight acute encephalitis cases and appraisal of niceties. J Infect Dev Ctries 30:517–30.

112. Sapkal, G.N., Bondre, V.P., Fulmali, P.V., Patil, P. et al. 2009. Enteroviruses in patients with acute encephalitis, Uttar Pradesh, India. Emerg Infect Dis 15:295–8.

113. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med. 2004; 10(Suppl): 98–109.

114. Huang, J.H., Lin, T.H., Teng, H.J., Su, C.L. et al. 2010, Molecular epidemiology of Japanese encephalitis virus, Taiwan. Emerg Infect Dis 16:876–8.

115. Dhillon, G.P and Raina,V.K. 2008. Epidemiology of Japanese Encephalitis in context with Indian scenario Indian Med Assoc (10):660-3.

116. Babu,G.N., Kalita, J. and Misra,U.K. 2006. Inflammatory markers in the patients of Japanese Encephalitis. Neu Res 28(2):90–192.

117. Ghosh, S.N., Rodrigues, F.M. and Seth, G.P. 1975. Investigations on the outbreak of Japanese encephalitis in Burdwan district, west Bengal. Part II. Serological survey of human population. In J Med Res 63(10):472–1477.

118. Rajagopalan, P.K. and Panicker, K.N. A note on the 1976 epidemic of Japanese Encephalitis in Burdwan district, west Bengal. J Medical Res 68:3938, 1978.

119. Rodrigues, F.M., Ghosh S.N. and Banerjee, K. 1975. A post epidemic serological survey of humans in Bankura district, west Bengal, following the epidemic of Japanese encephalitis in 1973, J of Med Res 63(10):1478–1485.

120. Vaughn, D.W., Hoke C.H. 1992. The epidemiology of Japanese encephalitis: prospects for prevention. Epidemiol Rev 14:197-221.

121. Debaratiguha, S., Barbara, S., 2005. Dengue fever: new paradigms for a changing epidemiology. Emer Themes Epi 2.1.

122. Nivedita, G., SakshiSrivastava., Amita J,. and Umesh C.C.,2012. Dengue in India. J Med Res 136(3):373–390.

123. Gubler, D.J. 2006. Denguehaemorrhagicfever:history and current status. Novartis found symp. 277:3-16.

124. Gubler, D. Dengue and Dengue hemorrhagic fever. 1998. ClinMicrobiol Rev 11(3):480-496.

125. Gubler D.J. Dengue and dengue hemorrhagic fever:its history and resurgence as a global public health problem. In Gubler D.J., Kuno G. editors. Dengue and Dengue hemorrhagic fever. London,CAB International 1-22.

126. Wilder smith, A. and Gubler, D.J. 2008. Geographic expansion of dengue:the impact of international travel Med Clin N Am 92(6):1377-1390.

127. WHO Regional office for South-East Asia. 2011. Comprehensive guidelines for prevention and control of Dengue and Dengue haemorrhagic fever. Revised and expand edition. New Delhi: world health organization South East Asia Regional office.

128. Gubler, D.J. 2002. Epidemic dengue/dengue/hemorrhagic fever as a public health, social and economic problem in the 21st century. TrenMicrobiol 10(2):100-103

129. Gubler, D.J. 2011. Dengue, urbanization and globalization:The unholy trinity of the 21stcentury. Trop Med Health 39(3):3-11.

130. Gubler, D.J., Clark, G.G. 1995. Dengue/dengue hemorrhagic fever: the emergence of a global health problem. Emerg Infect Dis 1(2):55–57.

131. World Health Organization TDR Global alert and response Dengue/Dengue haemorrhagic fever (webpage on the internet) Geneva: WHO 2013 (cited March 3). http://www.who.int/csr/disease/dengue/en/.

132. World Health Organization (WHO) Global strategy for Dengue prevention and control, 2012-2020. Geneva: WHO press:2012.

133. Natasha, E.A.M., Mikkel B.Q. and Annelies Wilder S. 2013. Epidemiology of dengue:past,present and future prospects. Clin epidemiol 5:299-309.

134. Smithburn K.C., Hughes T.P., Burke A.W. and Paul J.H. 1940. A neurotropic virus isolated from the blood of a native of Uganda. Am J Trop Med 20:471-92.

135. Peterson, L.R., roehrig J.T. 2001. West Nile virus: A re-emerging global pathogen. Emer Infect Dis 7:611-4.

136. Platonov, A.E., Shipuling, A., Shipulina, O.Y., Tyutyunnik, E.N. 1999. Outbreak of West Nile infection, vologograd region, Russia. Emerg Infect Dis 7:128-32.

137. Hubalek, Z., Halouzka, J. 1999. West Nile fecer- a re-emerging mosquito-borne viral disease in Europe. Emer Infect Dis 5:643-50.

138. Asnis, D.S., Conetta, R., Teixeria, A.A., Sampson B.A. 2000. The West Nile virus outbreak of 1999 in New York:the flushing hospital experience. Clin Infect Dis 30:413-8.

139. Weinberger, M., Pitlik, S.D., Gandacu, D., Lang, R. et al. 1999. West Nile outbreak, Israel.2000. Epidemiology aspects : Emerg infect Dis 2001 7:686-91.

140. Tsai, T.F., Popovici, F., Cernescu, C., Cambell, G.L. et al. 1998. West Nile encephalitis epidemic in south-eastern Romania. Lancet 352:767-71.

141. Update West Nile virus activity-north-eastern United States, January August 7, 2000: MMWR Morb Mortal Wkly Rep 49:714-8.

142. Gajanana, A., Thenmozhi, V., Samuel, P.P., Reuben, R. 1995. A community based study of sub clinical flavivirus infections in children in an endemic

area of Tamil Nadu, India, where Japanese encephalitis is endemic. Bull World Health Organization 73:237-44.

143. Albagal, C., Chaimoff, R. 1959. A case of West Nile myocarditis. Harcfach 57:274.974. Acute pancreatitis in West Nile fever. Am J Trop Med Hyg 23:1150-2.

144. Perelman, A., Stern, J. 1974. Acute pancreatitis in West Nile fever. Am J Trop Med Hyg 23:1150-2.

145. Work T.H. 1971. On the Japanese B West Nile virus complex or an arbovirus problem of six continents. Am Trop Med Hyg 20:169-86.

146. Huang, C., Slater, B. and Rudd, R. 2002. First isolation of West Nile virus from a patient with encephalitis in the United States. Emer Infect Dis 8:1367-71.

147. Schmidt, J.R., E, Mansoury, H.K. 1963. Natural and experimental infection of Egyptian equines with West Nile virus. Am Trop Med Parasitol 57:415-27.

148. Murgue, B., Murri, S., Zientara, S., Durand, B. et al. 2001. West Nile outbreak in horses in southern France, 2000. The return after 35 years. Emer Infect Dis 7:692-6.

149. Tber, A.A. 1996. West Nile fever in horses in Morocco. Bull office Int des Epizooties. 108:867-9.

150. Cantile, C., Di Guardo, G., Eleni, C., Arispici, M. 2000. Clinical and neuropathological features of West Nile virus equine encephalitis in Italy. Equine Vet J. 32:31-5.

151. Media centre 2014. Fact sheet No. 386. March. http://www.who.int/mediacentre/factsheet/sa386/en/.

152. Jairaj,P. Japanese encephalitis-an arboviral disease.http://www.who.int/csr/disease/dengue/en/

153. Ayan, G., Sulagna, D., Soumya G., Manojkumar, M. et al. 2006. Proinflammatory mediators released by activated microglia induces neuronal death in Japanese encephalitis. Wiley Inter Sci 55:483-496.

154. Gubler, D.J., Sather, G.E., Kuno G., Cabral, J.R. 1986. Dengue 3 virus transmission in Africa. Am J Trop Med Hyg 35(6):1280-1284.

155. Amarasinghe, A., Kuritsk, J.N., Letson, G.W., Margolis, H.S. 2011. Dengue virus infection in Africa. Emer infect Dis 17(8)1349-1354.

156. International travel and health dengue (web page on the internet)Geneva: WHO 2013.

157. Rigauperez, J.G., Clark, G.G., Gubler, D.J., Reiter P. et al. 1998. Dengue and dengue hemorrhagic fever. Lancet 352(9132):971-977.

158. Cao, X.T., Ngo N.T., Wills, B. et al. 2002. Evaluation of the WHO standard tourniquet test and a modified tourniquet test in the diagnosis of dengue infection in Vietnam. Trop Med Health 7:125-132.

159. Endy, T.P., Anderson, K.B., Nisalak, et al. 2001. Determinants of in apparent and symptomatic dengue infection in a prospective study of primary school children in KamphaengPhet, Thailand. PlosNegl Trop Dis 5(3):975.

160. Wildersmith ,A., Ooi, E.E., Vasudevan, S.G., Gubler, D.J. 2010. Update on dengue: epidemiology virus evolution, antiviral drugs and vaccine development. Curr Infect Dis Rep 12(3):157-164.

161. Wildersmith, S., Foo, W., Earnest, A., Sremulanathan, S. et al. 2004. Seroepidemiology of dengue in the adult population of Singapore. Trop med Int Health 9(2):305-308.

162. Gubler, D.J., The economic burden of dengue, 2012. Am J trop Med Hyg 86(5):743-744.

163. Roop, K. and Pyare, L.J. 2012. A review of Japanese encephalitis in Uttar Pradesh, India. J Public Health 1(4):374-395.

164. Kabilan, L., Vrati, S., Ramesh, S., Srinivasan,S. et al. 2004. Japanese encephalitis (JEV) is an important cause of encephalitis among children in Cuddalore district, Tamil Nadu, India. J clinvirol 31:153-9.

165. Mathur, A., Chaturvedi, U.C., Tandon, H.O. et al. 1982. Japanese encephalitis epidemic in Uttar Pradesh, India during 1978. In J Med Res 75:161-169.

166. Shailendra, K.S., Niraj Mishra., Rakhi, S., Maneesh, S. et al. 2009. Trend of Japanese encephalitis in North India:evidence from thirty-eight acute encephalitis cases and appraisal of niceties. J Infect Dev Ctries 3(7):517-530.

167. Kabilan, L., Rajendran, R., Arunachalam, N., Ramesh, S. et al. 2004. Japanese encephalitis in India: an overview. In J Pediatr 71(7):609-15.

168. Rao, B.L., Basu, A., Wairagkar, N.S., Miling, M.G. et al. A large outbreak of acute encephalitis with high fatality rate in children in Andhra Pradesh, India, in 2003, associated with Chandipura virus. Lancet 364:869-874.

169. Sarkar, J.K., Chatterjee, S.N., Chakravarty, S.K. 1964. Haemorrhagic fever in Calcutta: some epidemiological observations. In J Med Res 52:651-9.

170. Chatterjee, S.N., Chakravarti, S.K., Mitra, A.C., Sarkar, J.K. et al. 1965. Virological investigation of cases with neurological complications during the outbreak of haemorrhagic fever in Calcutta. J In Med Assoc 45:314-6.

171. Carey, D.E., Myers, R.M., Reuben, R., Rodrigues, F.M. 1966. Studies on dengue in Vellore, South India. In Am Trop Med Hyg 15:580-7.

172. Kabra ,S.K., Verma, I.C., Arora, N.K., Jain, Y. et al. 1992. Dengue haemorrhagic fever in children in Delhi. Bull WHO 70:105-8.

173. Bhattacharjee, N., Mukherjee, K.K., Chakravarti, S.K., Mukherjee M.K. 1990. Dengue haemorrhagic fever (DHF) outbreak in Calcutta. J Commun Dis 25:10-4.

174. Cherian, T., Ponnuraj, E., Kuruvilla, T., Kirubakaran, C. et al. 1994. An epidemic of dengue hemorrhagic fever & dengue shock syndrome in & around Vellore. J Med Res 100:51-6.

175. Dar, L., Broor S., Sengupta ,S., Xess, I. et al. 1999. The first major outbreak of dengue hemorrhagic fever in Delhi, India. Emerg Infect Dis 5:589-90.

176. Agarwal R., Kapoor, S., Nagar, R., Misra, A. et al. 1999. A clinical study of the patients with dengue hemorrhagic fever during the epidemic of 1996 at Lucknow, India. Southeast Asian J Trop Med Public Health 30:735-40.

177. Shah, I., Deshpande, G.C., Tardeja, P.N. 2004. Outbreak of dengue in Mumbai and predictive markers for dengue shock syndrome. J Trop Pediatr 50:301-5.

178. Karamchandani, P.V. 1946. Dengue group of fevers in India. Lancet. 1:92.

179. Sarkar, J.K., Pavri, K.M., Chatterjee, S.N., Chakravarty, S.K. et al. 1964. Virological and serological studies of cases of hemorrhagic fever in Calcutta. In J Med Res 52:684-91.

180. Chaudhuri, R.N., Saha, T.K., Chaudhuri, A.D. 1965. Dengue-like fever in Calcutta: further preliminary observations. Bull Calcutta Sch trop Med 13:2-3.

181. Krishnamurthy, K., Kasturi, T.E., Chittipantulu, G. 1965. Clinical and pathological studies of an outbreak of dengue-like illness in Visakhapatnam. In J Res 53:800-12.

182. Paul, S.D., Dandawate, C.N., Banerjee, K., Krishnamurthy, K. 1965. Virological and serological studies on an outbreak of dengue-like illness Vishakapatnam, Andhra Pradesh. In J Med Res 53:777-89.

183. Balaya, S., Paul, S.D., Dlima, L.V., Pavri, K.M. 1969. Investigation on an outbreak of dengue in Delhi in 1967. In J Med Res 57:767-74.

184. Chathrudvedi, U.C., Mathur, A., Kapoor, A.K., Mehrotra, N.K. et al. 1970. Virological study of an epidemic of febrile illness with hemorrhagic manifestations at Kanpur, India during 1968. Bull WHO 43:289-93

185. Chathruvedi, U.C., Kappor, A.K., Mathur, A., Chandra, D. et al 1970. A clinical and epidemiological study of an epidemic of febrile illness with hemorrhagic manifestations which occurred at Kanpur, India in 1968. Bull WHO 43:281-7.

186. Myers, R.M., Carey, D.E., Banerjee, K., Reuben, R. et al. 1968. Recovery of dengue type 3 virus from human serum and *Aedes aegypti* in South India. J Med Res 56:781-7.

187. Ghosh, B.N. 1968. A study on the epidemic of dengue-like fever in Pondicherry (1964and1965). Med Assoc 51:261-4.

188. Mahadev, P.V., Kollai, V.V., Rawal, M.L., Pujara, P.K. et al. 19993. Dengue in Gujarat state, India during 1988& 1989. J Med Res 97:135-44.

189. Ghosh, S.N., Pavri, K.M., Singh, K.R., Sheikh B.H. et al. 1974. Investigations on the outbreak of dengue fever in Ajmer city, Rajasthan state in 1969. Part I. epidemiological, clinical and virological study of the epidemic. In J Med Res 62:511-22.

190. Chouhan, G.S., Rodrigues, F.M., Shaikh, B.H., Ilkal M.A. et al. 1990. Clinical and virological study of dengue fever outbreak in Jalore city, Rajasthan 1985. J Med Res 91:414-8.

191. Rodrigues, F.M., Pavri, K.M., Dandawate, C.N., Banerjee K. et al. 1973. An investigation of the aetiology of the 1966 outbreak of febrile illness in Jabalpur, Madhya Pradesh. J Med Res 61:1462-70.

192. Kumar, A., Sharma, S.K., Padbidri, V.S., Thakare, J.P. et al. 2001. An outbreak of dengue fever in rural areas of northern India. J Commun Dis 33:274-81.

193. Parade, M.M., Dash, P.K., Upadhyay C., Saxena, P. et al. 2002. Serological and virological investigation of an outbreak of dengue fever in Gwalior, India. J Med Res 116:248-54.

194. Kurukumbi, M., Wali, J.P., Broor, S., Agarwal, P. et al. 2001. Seroepidemiology and active surveillance of dengue fever/dengue hemorrhagic fever in Delhi. J Med Sci 55:149-56.

195. Singh, U.B., Maitra, A., Broor, S., Rai, A. et al. 1999. Partial nucleotide sequencing and molecular evolution of epidemic causing dengue 2 strains, J Infect Dis 180:959-65.

196. George, S., Prasad, S.R., Rao J.A., Yergolkar, P.N. et al. 1987. Isolation of Japanese encephalitis and West Nile viruses from fatal cases of encephalitis in kola district of Karnataka. J Med Res 86:131.

197. Anukumar, B., Sapkal, G.N., Tandale, B.V., Balasubramanian, R. et al. 2014. West Nile encephalitis outbreak in Kerala, India 2011. J ClinVirol 61(1):152-5.

198. Igarashi, A., Morita, D. 1994. Detection of West Nile and Japanese encephalitis viral genome sequences in cerebrospinal fluid from acute encephalitis cases in Karachi, Pakistan. Microbiol Immunol 38:827-30.

199. Palmer, D.F., Whaley, S.D. 1986. Complement fixation test. In rose NR, Friedman H., Fahey J.L., editors. Manual of Clin Lab immunol (third edition), Washington DC, Am Soci Microbiol 57-66.

200. Monath, T. Flaviviruses (Yellow fever, dengue, dengue haemorrhagic fever, Japanese encephalitis, St. Louis encephalitis, tick-borne encephalitis. In Mandell G.G., Bennett J.E., Dolin R. editors. Principles and practice of Infectious Diseases. New York, Churchill Livingston Inc.1465-74.

201. Kuno, G. 2003. Serodiagnosis of flaviviral infections and vaccinations in humans. Adv Virus Res 61:3-65.

202. Russel, P.K., Nisalak, A., Sukhavachana, P., Vivona S. 1967. A plaque reduction test for neutralizing antibodies. J Immunol 99:285-90.

203. Gadkari, D.A., Shaikh, B.H. 1984. IgM antibodies capture ELISA in the diagnosis of Japanese encephalitis, West Nile and Dengue virus infections. J Med Res 80:613-9.

204. Martin, D.A., Muth, D.A., Brown T., et al. 2000. Standardization of immunoglobulin Captureenzyme-linked immunosorbents assays for routine diagnosis of arboviral infections. J Clin Microbiol 38:1823-6.

205. Shi, P.Y., Wong, S.J. 2003. Serologic diagnosis of West Nile virus infection. Expert Rev MolDiagn 2:733–41.

206. Matthias, N., Oliverkur, S., Christian, H., Karen, S. 2008. Evaluation of an indirect immunofluorescence assay for detection of Immunoglobulin (IgM) and IgG antibodies against Yellow fever virus. Clin and Vaccine Immunology 15(2):177-181.

207. Martins, T.B., Jaskowski, T.D., Mouritsen, C.L., Hill H. An evaluation of the effectiveness of three Immunoglobulin G (IgG) removal procedures for routine IgM serological testing. Clin Diagn Lab Immunol 2:98–103.

208. Huang, C., Slater, B., Campbell, W. et al. Detection of arboviral RNA directly from mosquito homogenates by reverse-transcription polymerase chain reaction. J Virol Methods 94:121–8.

209. Hindiyeh, M., Shulman, L.M., Mendelson, E. et al. 2001. Isolation and characterization of West Nile virus from the blood of viremic patients during the 2000 outbreak in Israel. Emer Infect Dis 7:748–50.

210. Tilley, P.A., Fox J.D., Jayaraman, G.C. et al. 2006. Nucleic acid testing for West Nile virus RNA in plasma enhances rapid diagnosis of acute infection in symptomatic patients. J Infect Dis 193:1361–4.

211. Lanciotti, R.S., Kerst, A.J., Nasci, R.S. et al. 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and

avian samples by a Taman reverse transcriptase-PCR assay. J ClinMicrobiol 38:4066–71.

212. Weiss, D., Carr, D., Kellachan J. et al. 2000. Clinical findings of West Nile virus infection in hospitalized patients, New York and New Jersey. Emerg Infect Dis 7: 654–8.

213. Hiatt, B., DesJardin L., Carter, T. et al. A fatal case of West Nile virus infection in a bone marrow transplant recipient. Clin Infect Dis 37:129–31.

214. Huang, C., Slater, B., Rudd, R. et al. 2002. First isolation of West Nile virus from a patient with encephalitis in the United States. Emer Infect Dis 8:1367-71.

215. Busch, M.P., Caglioti, S., Robertson, E.F. et al. 2005. Screening the blood supply for West Nile virus RNA by nucleic acid amplification testing. N Engl J Med 353:460-67.

216. Lanciotti, R.S., Kerst, A.J., Nasci, R.S. et al. 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol 38:4066–71.

217. Johnson, D.J., Ostlund, E.N., Pedersen, D.D., Schmitt, B.J. 2001. Detection of North American West Nile virus in animal tissue by a reverse transcription-nested polymerase chain reaction assay. Emerging Infectious Disease 7: 739-41.

218. Shi, P.Y., Kauffman, E.B., Ren P., Felton, A. et al. 2001. High- throughput detection of West Nile virus RNA. J Clin Microbiol 39:1264-71.

219. Lanciotti, R.S., Kerst A.J. 2001. Nucleic acid sequence- based amplification assays for rapid detection of West Nile and St. Louis encephalitis viruses. J ClinMicrobiol 39:4506-13.

220. Scott, B.H. 2012. Molecular virology and control of Flavivirus. Flaviviruses vaccines. 185-186.

221. Vaughn D.W., and Hoke C.H. 1992. The epidemiology of Japanese encephalitis: prospects for prevention. Epidemiol Rev14:197-221.

222. Lanciotti , R. S., Roehrig, J. T., Dubrl, V., Smith, J. et al. 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the NorthEastern United states. Science 286:2333-2337.

223. Mackenzie, J.S., GublerD.J.and Peterson L.R. 2004. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med 10:S98-S109.

224. Mackenzie, J. S.2005. Emerging zoonotic encephalitis viruses: lessons from Southeast Asia and Oceania. J Neurovirol 11:434-440.

225. Halstead, S.B. 1988. Pathogenesis of dengue: challenges to molecular biology. Science 239:476-481.

226. Kanai, R., Kirk. Anthony K., Gould L.H. et al. 2006. Crystal structure of West Nile virus envelope glycoprotein reveals viral surface epitomes. JVirol 80:11000-11008.

227. Rey,F. A., Heinz, F. X., Mandl, C., Kunz, C. 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolutions. Nature 375:291-298.

228. Crill, W. D. and Chang, G. J.2004. Localization and characterization of flavivirus envelope glycoprotein cross-reactive epitopes. JVirol 78:13975-13986.

229. Roehrig, J.T., Bolin, R.A. and Kelly, R.G. 1998. Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica. Virology 246:317-328.

230. Roehrig, J.T., Hunt, A.R., Johnson, A.J. and Hawkes R. A. 1989. Synthetic peptides derived from the deduced amino acid sequence of the E-glycoprotein of Murray Valley encephalitis virus elicit antiviral antibody. Virology 171:49-60.

231. Roehrig, J.T., Johnson, A. J., Hunt, A.R., Bolin, R.A. et al. 1990. Antibodies to dengue 2 virus E-glycoprotein synthetic peptides identify antigenic conformation. Virology 177:668-675.

232. Roehrig, J.T., Mathews, J.H. and Trent. 1983. Identification of epitopes on the E glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. Virology 128:118-126.

233. Stiasny, K., Kiermayr, S., Holzmann, H. and Heinz, F.X. 2006. Cryptic properties of a cluster of dominant flavivirus cross-reactive antigenic sites. J Virol 80:9557-9568.

234. Sukupolvi-Petty S., Austin, S.K., Purtha, W.E., Oliphant, T. et al. 2007. Type- and sub complex-specific neutralizing antibodies against domain III of dengue virus type 2 envelope protein recognize adjacent epitopes. J Virol 81:12816-12826.

235. Martina, B.R., Koraka, P. and Osterhaus, a.d., 2009. Dengue virus pathogenesis: an integrated view. ClinMicorbiol Rev 22(4):564-581.

236. Vasilakis, N., ad weaver, S.C. 2008. The history and evolution of human dengue emergence. Adv Virus Res 72:1-76.

237. Hunspergerr, E.A., Yoksan, S., Buchy, P. 2009. Evaluation of commercially available anti-dengue virus immunoglobulin M tests. Emer Infect Dis 15(3):436-440.

238. Depaula, S.O., fonseca, B.A. 2004. Dengue: a review of the laboratory tests a clinician must know to achieve a correct diagnosis. Braz J Infect Dis 8(6):390-398.

239. Ayers, M., Adachi, D., Johnson, G., Andonova, M. et al. A single tube RT-PCR assay for the detection of mosquito-borne flaviviruses. J Viro Methods135(2): 235–239.

240. Grant-Klein, R.J., Baldwin, C.D., Turell, M.J. 2010. Rapid identification of vector-borne flaviviruses by mass spectrometry.Mol Cell Probes24(4): 219–228.

241. Johnson, N., Wakeley, P.R., Mansfield, K.L. 2010. Assessment of a novel real-time pan-flavivirus RT-polymerase chain reaction. Vector Borne Zoonotic Dis10(7): 665–671.

242. Maher-Sturgess, S.L., Forrester, N.L., Wayper, P.J. 2008. Universal primers that amplify RNA from all three flavivirus subgroups. Virol J 5: 16.

243. Moureau ,G., Temmam ,S., Gonzalez ,J.P., Charrel, R.N. et al. 2007. A real-time RT-PCR method for the universal detection and identification of flaviviruses. Vector Borne Zoonotic Dis 7(4): 467-477.

244. Chao, D.Y., Davis, B.S., Chang, G.J. 2007. Development of multiplex real-time reverse transcriptase PCR assays for detecting eight medically important flaviviruses in mosquitoes. J ClinMicrobiol 45(2): 584–589.

245. Chow, V.T., Seah, C.L., Chan ,Y.C. 1993. Use of NS3 consensus primers for the polymerase chain reaction amplification and sequencing of dengue viruses and other flaviviruses. Arch Virol 133(1–2): 157–170.

246. Dyer, J., Chisenhall, D.M., Mores, C.N. 2007. A multiplexed TaqMan assay for the detection of arthropod-borne flaviviruses. J Virol Methods 145(1): 9–13.

247. Eldadah, Z.A., Asher, D.M. 1991. Godec MS et al. Detection of flaviviruses by reverse-transcriptase polymerase chain reaction. J Med Virol 33(4): 260–267.

248. Figueiredo, L.T., Batista, W.C., Kashima, S., Nassar, E.S. 1998. Identification of Brazilian flaviviruses by a simplified reverse transcription-polymerase chain reaction method using flavivirus universal primers. Am J Trop Med Hyg 59(3): 357–362.

249. Gaunt, M.W., Gould, E.A. 2005. Rapid subgroup identification of the flaviviruses using degenerate primer E-gene RT-PCR and site specific restriction enzyme analysis. JVirol Methods 128(1–2): 113–127.

250. Kuno, G., Chang, G.J., Tsuchiya, K.R., Karabatsos, N. et al. 1998. Phylogeny of the genus Flavivirus. J Virol 72(1): 73–83.

251. Puri, B., Henchal, E.A., Burans, J. 1994. A rapid method for detection and identification of flaviviruses by polymerase chain reaction and nucleic acid hybridization. Arch. Virol.134 (1–2): 29–37.

252. Sanchez-Seco, M.P., Rosario, D., Domingo, C. 2005. Generic RT-nested-PCR for detection of flaviviruses using degenerated primers and internal control followed by sequencing for specific identification. J VirolMethods126(1–2): 101–109.

253. Scaramozzino, N., Crance, J.M., Jouan, A., DeBriel, D.A. et al. 2001. Comparison of flavivirus universal primer pairs and development of a rapid, highly sensitive hemi nested reverse transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the NS5 gene sequences. J ClinMicrobiol 39(5): 1922–1927.

254. Tanaka, M. 1993. Rapid identification of flavivirus using the polymerase chain reaction. J Virol Methods 41(3): 311–322.

255. Scaramozzino, N., Crance, J.M., Jouan, A., DeBriel, D.A.Stoll, F, Garin, D. et al. 2001. Comparison of flavivirus universal primer pairs and development of a rapid, highly sensitive heminested reverse transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the NS5 gene sequences. J ClinMicrobiol 39(5): 922–1927.

256. Das, S., Pingle, M.R., Munoz-Jordan, J. 2008. Detection and serotyping of dengue virus in serum samples by multiplex reverse transcriptase PCR-ligase detection reaction assay. J ClinMicrobiol 46(10): 3276–3284.

257. Yeh, J.Y., Lee, J.H., Seo, H.J. 2010. Fast duplex one-step reverse transcriptase PCR for rapid differential detection of West Nile and Japanese encephalitis viruses. J ClinMicrobiol 48(11): 4010–4014.

258. Hull, R., Nattanmai, S., Kramer, L.D., Bernard, K.A. and Tavakoli, N.P. 2008. A duplex real-time reverse transcriptase polymerase chain reaction assay for the detection of St. Louis encephalitis and eastern equine encephalitis viruses. DiagnMicrobiol Infect Dis 62(3): 272–279.

259. Yang, C.F., Chen, C.F., Su, C.L. et al. 2010.Screening of mosquitoes using SYBR Green I-based real-time RT-PCR with group-specific primers for detection of flaviviruses and alphaviruses in Taiwan. J Virol Methods 168(1–2): 147–151.

260. Naze, F., Le Roux, K., Schuffenecker, I. et al. 2009. Simultaneous detection and quantitation of Chikungunya, dengue and West Nile viruses by multiplex RT-PCR assays and dengue virus typing using high resolution melting. JVirol Methods 162(1–2): 1–7.

261. Rondini, S., Pingle, M.R., Das, S. et al. 2008. Development of multiplex PCR-ligase detection reaction assay for detection of West Nile virus. JClinMicrobiol 46(7): 2269–2279.

262. EI Bahnasawy, M.M., Khater, M.K. and Morsy, T.A. 2013. The, mosquito borne West Nile virus infection: is it threating to Egypt or a neglected endemic desease. J Egypt SocParasitol 43(1):87-102.

263. Tamura, K., Dudley J. Nei M., Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics (MEGA) software, version 4.0. Mol Biol Evol 24:1596–9.

264. Kao, C., King, C., Chao, D., Wu, H. et al. 2005. Laboratory diagnosis of dengue virus infection:current and future perspectives in clinical diagnosis and public health. J MicrobiolImmunol Infect 38:5-16.

265. Shu, P.Y., Huang, J.H. 2004. Current advances in dengue diagnosis. ClinDiagn Lab Immunol 11:642-50.

266. Pattnaik, P., Srivastava, A., Abhyankar, A., Dash, P.K. et al., 2006. Fusogenic peptide as diagnostic marker for detection of fusogenic peptide as diagnostic marker for detection of flaviviruses. J Postgrad Med 52:174-8.

267. Shigeki, M., Yoshiyuki, T., Shigeru ,Y., Isao, F. et al. 1994. Highly sensitive detection of viral RNA genomes in blood specimens by an optimized reverse transcription-polymerase chain reaction. J Med Virol 43:175-181.

268. Robert, S.L., Charles, H.C., Duane, J.G., Gwongjen C., et al. 1992. Rapid detection and typing of Dengue viruses from clinical samples by using reverse transcriptase polymerase chain reaction. J ClinMicrobiol 30(3):545-551.

269. Thuraiayah, V., Kirk, M., Michael, D. and Roger, H. 2005. Molecular typing of West Nile, Dengue and St. Louis encephalitis using multiplex sequencing. J Molecular Diag 7(2):152-159.

270. Guidelines for the production of antibodies in laboratory animals. 2014. Animal care and use program. University of California, Berkeley. http://www.acuc.berkeley.edu/guidelines/antibodies.

271. Beth, K. S., Nora, M.C. and Peter C.I. 2009. Overview of the Flaviviridae with an emphasis on the Japanese encephalitis group viruses. Lab Med 40:493-499.

272. Norris, P. and Inglehart, R. 2003. Rising Tide: Gender Equality and Cultural Change around the World. New York: Cambridge University Press.

273. Gregory, J.M., Ingram, W.J., Palmer, M.A., Jones, G.S. et al. 2004. A new method for diagnosing radioactive forcing and climate sensitivity. Geographical research letters. 31(3): Feb.

274. Johnson, B.W., Kosoy, O., Martin, D.A., Noga, A.J. et al. 2005. West Nile virus infection and serological response among persons previously

vaccinated against Yellow fever and Japanese encephalitis viruses. Vector Borne Zoonotic Dis 5(2):137-45.

275. Solomon, T., Dung, N.M., Vaughn, D.W., Kneen, R. et al. 2000. Neurological manifestations of dengue infection. Lancet 355:1053-9.

276. Solomon, T. 2004. Flavivirus encephalitis. N Engl J Med 351(4):370-378.

277. Upadhyayula, S.M., Rao, M.S., Nayanoori, H.K., Natarajan, A. et al. 2012. Impact of weather variables on mosquitoes infected with Japanese encephalitis virus in Kurnool district, Andhra Pradesh. Asian Pac J Trop Med 5:337–341.

278. Sarkar, A., Taraphdar, D., Mukhopadhyay, S.K., Chakrabarti, S. et al. 2012. Serological and molecular diagnosis of Japanese encephalitis reveals an increasing public health problem in the state of West Bengal, India. Trans R Soc Trop Med Hyg106(1):15–19.

279. Githeko, A.K., Lindsay S.W., Confalonieri, U.E. and Patz, J.A. 2000. Climate change and vector-borne disease: a regional analysis. Bull. World Health Org 78:1136-1147.

280. Murthy, H.M., Judge, K., DeLucas, L. and Padmanabhan, R. 2000. J Mo. Biol 301:759-767.

281. Karunaratne, S.H.P.P., Hemingway, J., Jayawardena, K.G.I., Dassanayaka, V. et al. 1995. Kinetic and molecular differences in the amplified and non-amplified esterases from insecticide resistant and susceptible Culex quinquefasciatus mosquitoes. J Biological Chemistry 270:31124–31128.

282. Debapriya, G. and Anirban B. 2009. Japanese encephalitis-a pathological and clinical perspective. PlosNegl Trop Dis 3(9):437.

283. Jamaluddain, M. and Saxena, V.K. 1997. First outbreak of Dengue fever in a typical rural area of Haryana state in northern India. J commun Dis 29(2):169-70.

284. Ronan Kelly. 2014. Acute encephalitis syndrome outbreaks in India-an ongoing puzzle. School of public health and community medicine. https://sphcm.med.unsw.edu.au/infectious-diseases-blog

285. Gould, E. A., Solomon, T. and Mackenzie, J.S. 2008. Does antiviral therapy have a role in the control of Japanese Encephalitis? Antiviral Research 78(1):140-149.

286. Diagana, P.M., Preux and Dumas M. 2007. Japanese encephalitis revisited. J Neurological Sci 262(1-2):165–170.

287. Murty, U.S., Rao, M.S. and Arunachalam, N. 2010. The effects of climatic factors on the distribution and abundance of Japanese encephalitis vectors in Kumool district of Andhra Pradesh, India. J Vector Borne Dis 47:26-32.

288. Kavita, R. 2007. Dengue fever: The rise and establishment of a new disease in Kerala, India with special references to the capital, Thiruvananthapuram. J Acad Clin Microbiol. 9:65–70.

289. Sucre, N.C., Liras, K., Wanda, T., Did, S. et al. 2003. Transmission of epidemic dengue hemorrhagic fever in easternmost Indonesia. Am J Trop Med Hyg 68:529-35.

290. Gag, A., Gag, J., Rao, Y.K., Upadhyay, G.C. et al. 2011. Prevalence of dengue among clinically suspected febrile episodes at a teaching hospital in North India. J Infect Dis Immun. 3:85-9.

291. Chakravarti, A. and Kumaria, R. 2005. Eco-epidemiological analysis of dengue infection during an outbreak of dengue fever, India. Virol J 2:32.

292. Sharma, Y., Kaur M., Singh, S., Pant, L. et al. 2012. Seroprevalence and trend of dengue cases admitted to a Government hospital, Delhi- 5year Study (2006-2010): A look into the age shift. Int J Prev Med 3:537-43.

293. Martina, B.E., Koraka, P., Osterhaus, A. 2009. Dengue virus pathogenesis, an integrated view. ClinMicrobiol Rev 22:564-81.

294. Gunasekaran, P., Kaveri, K., Mohana, S., Arunagiri, K. et al. 2011. Dengue disease status in Chennai (2006-2008): A retrospective analysis. Indian J Med Res 133:322-5.

295. Papin, J.F., Vahrson, W., Dittmer, D.P. 2004. SYBR Green-based real-time quantitative PCR assay for detection of West Nile virus circumvents false-negative results due to strain variability. J ClinMicrobiol 42:1511–8.

296. Vanlandingham, D.L., Schneider, B.S., Klingler, K., Fair, J. et al. 2004. Real-time reverse transcriptase polymerase chain reaction quantification of West Nile virus transmitted by Culex pipiens, quinquefasciatus. Am J Trop Med Hyg 71:120-3.

297. Jimenez-Clavero, M.A., Aguero, M., Rojo, G., Gomez-Tejedor, C. 2006. A new fluorogenic real-time RT-PCR assay for detection of lineage 1 and lineage 2 West Nile viruses. J Vet Diag Invest 18:459-62.

298. Clarke, D.H. and Casals J. 1958. Techniques for hemagglutination and hemagglutination inhibition with arthropod-borne viruses. AmJ Trop Med Hyg 7(5):561-73.

299. Charul Gijavanekar, Mariam, A.L., Chenm, F, Catherine , P. et al. 2010. PCR detection of nearly any Dengue virus strain using a highly sensitive primer cocktail. FEBS J 278:1676-1687.

300. Campbell G.L., Hills S.L., Fischer M., Jacobson J.A. et al. 2011. Estimated global incidence of Japanese encephalitis; a systematic review. Bull WHO 89:766-774, 774A-774E.

301. Japanese encephalitis surveillance standards. From WHO recommended standards for surveillanceofselectedvaccine preventable diseases. http://WWW.path.org/Vaccinesources/files/WHO-JE-surveillance stsndards.pdf.

302. WHO. 2010. Internationsl travel and Health WWW.who.int/ith/IYH2010.

303. Diagana, M., Preux P.M. and Dumas, M. 2007. Japanese encephalitis revisited. J NeurolSci 262:165-170.

304. Scherer, W.F., Kitaoka, M., Okuno, T. and Ogata, T. Ecologic studies of Japanese encephalitis virus Japan. VII Human infection. Am J Trop Med Hyg 8:707-715.

305. Namachivayam, V. and Umayalk, K. 1982. Proceedings of the National Conference on Japanese Encephalitis, ICMR New Delhi, India.

306. Vandana, S. and Tapan, N.D. 2008. Preventive strategies for frequent outbreaks of Japanese encephalitis in northern India. J Bio Sci 33:505-514.